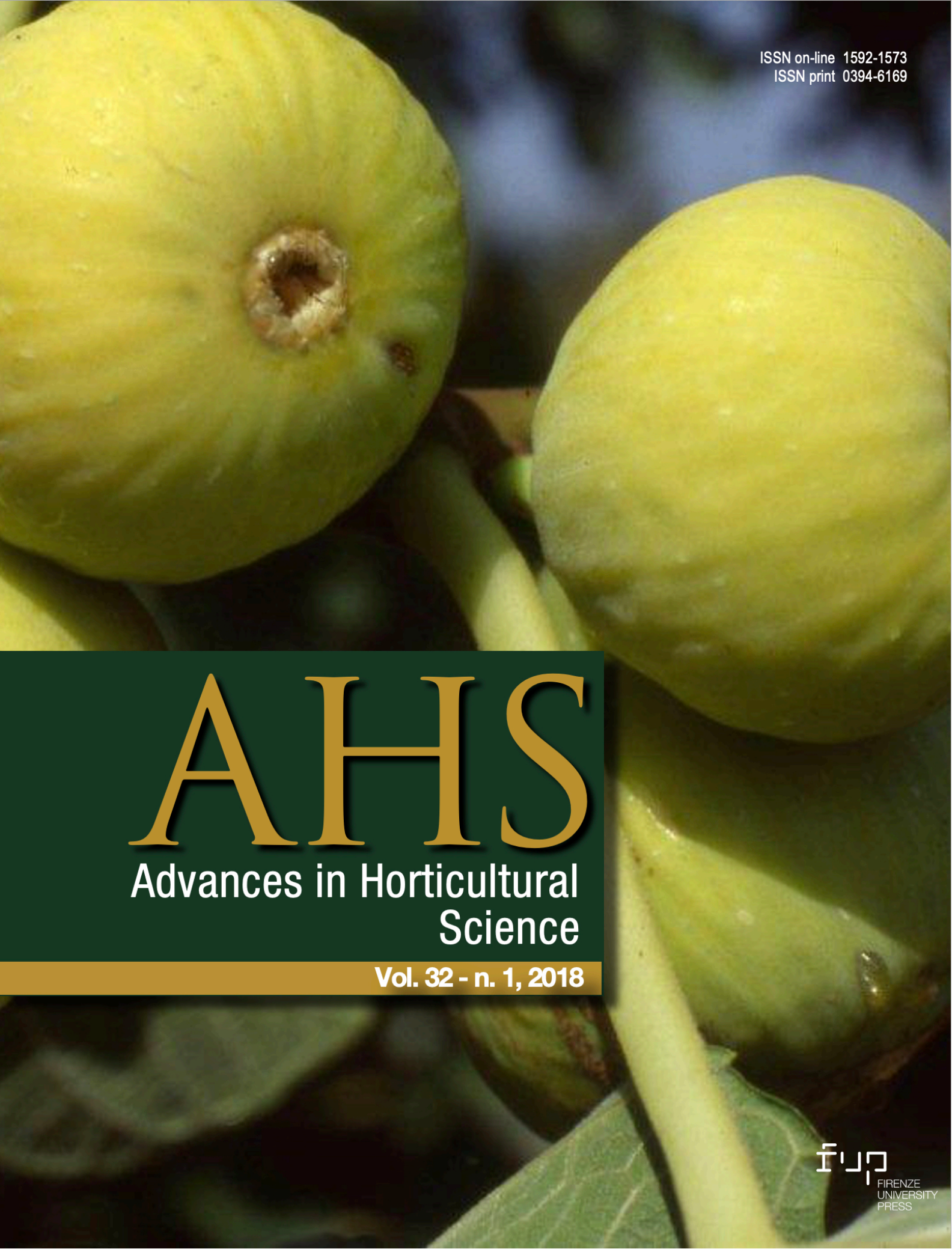
ISSN on-line 1592-1573
ISSN print 0394-6169



# AHS

## Advances in Horticultural Science

Vol. 32 - n. 1, 2018

FIRENZE UNIVERSITY PRESS

***Advances in Horticultural Science***
*Published by* **Firenze University Press** - University of Florence, Italy
Via Cittadella, 7 - 50144 Florence - Italy
http://www.fupress.com/ahs

Direttore Responsabile: **Francesco Ferrini**, University of Florence, Italy.


**Cover:** Reworked image from the DAGRI archive.

# ADVANCES IN HORTICULTURAL SCIENCE

Formerly Rivista dell'Ortoflorofrutticoltura Italiana
founded in 1876 and issued by University of Florence, Italy

supported by ENTE CASSA DI RISPARMIO DI FIRENZE

**Advances in Horticultural Science is covered in the following indexing and abstracting services:**

ACNP Catalogo Italiano dei Periodici - AGRICOLA - AGRICULTURE Journals - AGRIS -APE Journal - BASE Bielefeld Academic Search Engine - BIOBASE - Biological Abstracts - Biological Science Journals PQ - BIOSIS Preview THOMSON REUTERS - CAB Abstracts - EBSCO - EZB Elektronische Zeitschriften Bibliothek - Universitäts bibliothek Regensburg - Google Scholar - HORTICULTURAL Abstracts -Journal Seek. A Searchable Database of Online Scholarly Journals - JURN - Natural Science Journals PQ - NewJour. Electronic Journals & Newsletters, University of Georgetown - OAISTER oclc - Ornamental Horticulture CABI - Plant Breeding Abstract CABI - Proquest - Scirus ELSEVIER - SciTech Journals - SciVerse SCOPUS ELSEVIER - Searchteam - Ulrich's Periodicals Directory - WoS Web of Science THOMSON REUTERS - WorldCat

Advances in Horticultural Science is published by the Department of Agri-Food Production and Environmental Sciences, University of Florence, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy
Phone +39-055-4574021-22, Fax +39-055-4574910, E-mail: advances@dispaa.unifi.it

**CONTENTS**

*SHORT NOTES*

AHS
Advances in Horticultural Science

# 24-Epibrassinolide improves some physiological disorders in pistachio cultivars

**F. Kamiab** (*)
*Department of Horticulture, Rafsanjan Branch, Islamic Azad University, Rafsanjan, Iran.*

*Key words*: abscission, antioxidant enzymes, hormone, physiological disorders, spraying.



(*) **Corresponding author:**
f.kamiab56@gmail.com

**Citation:**
KAMIAB F., 2018 - *24-Epibrassinolide improves some physiological disorders in pistachio cultivars.* - Adv. Hort. Sci., 32(1): 3-12



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 18 July 2017
Accepted for publication 31 October 2017

**Abstract: The effect of 24-epibrassinolide foliar spray on some physiological disorders of pistachio cultivars was evaluated in two years. This factorial experiment was designed as a randomized complete block design with three replications. Factor A involved different pistachio cultivars and factor B involved the application time of hormone with four treatments including T1= bud swelling, T2= after full bloom, T3= T1 + T2, and T4= control. Different parameters were recorded. The application of hormone in both different stages caused the maximum fruit set percentage and minimum inflorescence bud and fruit abscission percentage in three sprayed cultivars. Despite of the highest percentage of inflorescence bud and fruit abscission in control treatment of cv. Kallehghoochi, the application of this hormone decreased these parameters in this cultivar in T3 treatment. Chlorophyll, protein, and proline contents of the leaves were increased in all brassinosteroide treatments, especially under two-stage application. Also, ion leakage and reduced sugar were decreased in treated leaves after using hormones. The effect of this hormone on antioxidant enzymes showed that all enzymes (CAT, SOD and APX) except POD were increased by the application of brassinosteroides. The loss of carbohydrate in one-year-old woods indicated the growth stimulating effect of this hormone. Thus, 24-epibrassinolide is a good suggestion to increase the quantity and quality of pistachio, especially in cv. Kallehghoochi.**

## 1. Introduction

Pistachio (*Pistacia vera* L.) is an important horticultural crop that has high economic value. Unfavourable environmental conditions in recent years have resulted in the loss of fruit set and yield in commercial pistachio cultivars. Also, physiological disorders such as bud and fruit abscission, and blank and non-split nuts are the most important problems of this produce that have been deteriorated by the biotic and abiotic stresses in most pistachio-growing areas.

Plant antioxidant defence system consists of such enzymes as superox-

ide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and also, non-enzymatic components may include osmolytes like proline, glycine betaine, sorbital, and mannitol (Anjum *et al.*, 2010, 2012).

In these conditions, growth regulators are useful for the prevention of fruit abscission and increased yield. Nowadays, foliar spray of growth regulators in fruit orchards has become a usual practice, and their considerable effects are evident on fruit quantitative characteristics. Brassinosteroids (BRs) are steroid hormones that are widely distributed in the plant kingdom, with a regulatory function in normal plant growth and development (Mandava, 1988).

Exogenous application of BRs to roots of young tomato and radish plants induced the hypocotyls and petioles (Takatsuto *et al.*, 1983). Brassinosteroids are known as hormones with pleiotropic effects that influence different processes like cell elongation, senescence and xylem differentiation, growth, seed germination, rhizogenesis, flowering, stem elongation, pollen tube growth, leaf epinasty, ethylene biosynthesis, proton pump activation, gene expression, and photosynthesis (Clouse and Sasse, 1998; Dhaubhadel *et al*., 1999; Khripach *et al*., 2000; Steber and McCourt, 2001; Krishna, 2003; Yu *et al*., 2004; Vert *et al.*, 2006). Brassinosteroids also could induce resistance to various abiotic stresses (Rao *et al.*, 2002).

Commercial application of this hormone has been started since 1990 and 24-epiprassinolid and 28-homobrassinolid are more effective in garden conditions because of their stability (Khripach *et al.*, 2000). Effects of 28-homobrassinolide have been reported on yields of wheat, rice, groundnut, mustard potato, and cotton (Ramraj *et al.*, 1997). Foliar spray of brassinoides on watermelon seedling increased female flower, fruit set and yield considerably. In other experiments, effective role of brassinosteroid has been confirmed in vegetative and reproductive growth in strawberries and yellow passion fruit (Ramraj *et al.*, 1997; Gomes *et al.*, 2006). Also, the application of BRs could accelerate the ripening of tomato and grape fruits (Vardhini and Rao, 2002; Symons *et al*., 2006). Despite numerous studies on agronomic crops, there are few investigations into the effect of brassinosteroides on fruit trees.

Hosseinpur *et al.* (2014) used different concentrations (0.5, 0.75, 1 and 1.5 mg/l) of 24-epibrassinolid before flowering on Kallehghuchi pistachio and showed that 1.5 mg/l of epibrassinolid was the best treatment for increasing fruit set of this cultivar. Due to the increasing unfavourable environmental conditions during pistachio flowering, this hormone was tested before and after flowering for increasing fruit set. Numerous studied has showed that effect of this hormone depends on its application time. Thus, we compared different application times on three commercial cultivars to determine the best application time for brassinosteroides on pistachio trees. The objective of the study was to evaluate the effect of 24-epibrassinolid on quantitative and qualitative traits of pistachio fruit.

## 2. Materials and Methods

*Plant material and experiments*

This experiment was done in a commercial orchard (Lat. 29°30' N., Long. 56°05' E., Alt. 1605 m.) in the Asad Abad district, Rafsanjan, Iran in 2014 and 2015. The results of soil analysis of this orchard are shown in Table 1. Mineral deficiencies are compensated with the use of fertilizer during the growing season.

In 2014, twenty adjacent "on" trees were selected. On each "on" tree, five uniforms "on" shoots were chosen and labeled a week before full bloom. Each selected shoot included four clusters with no lateral shoots. Because of the alternate bearing habit of pistachio trees, "on" trees in 2014 were naturally "off" trees in 2015, so this experiment was repeated by applying the same treatments on the similar previous year's trees, naturally going into the "off" year, but the treatments were applied on other one-year-old shoots to avoid any experimental effects of the previous year's treatments. These selected shoots were also uniform in length and diameter similar to the previous year's shoots, but each shoot included only one cluster. 24-epibrassinolide (1.5 mg/l, Sigma

Table 1 - Chemical analysis of some macro and micro elements of the soil of the pistachio orchard in Asad Abad village, Rafsanjan

| Texture soil | pH | Saturation (%) | EC (ds/m) | Depth (cm) | K (ppm) | P (ppm) | N (%) |
|---|---|---|---|---|---|---|---|
| Sandy-clay | 7.7 | 28 | 7 | 0-30 | 239 | 13.8 | 0.03 |

Company) was sprayed on crown of the trees.

The experiment was carried out on a factorial base with a randomized complete block design with three replications. Factor A involved different pistachio cultivars (Akbari, Kalehghoochi and Ohadi). Factor B involved the time of the application of 24-epibrassinolide in four treatments: T1= bud swelling stage, T2= two weeks after full bloom, T3= application at two stages (T1 and T2) and T4= control (spraying with water). There were 12 treatments with 3 replications in this experiment, and two trees in each experimental unit (totally 72 trees) were compared.

*Inflorescence bud and fruit abscission and fruit set*

The number of initiated inflorescence buds and the total number of abscised buds on the individual current-year shoots were counted six weeks after full bloom (May) and at the harvest time (September), respectively. The percentage of inflorescence bud abscission was calculated by dividing the number of abscised buds by the total number of buds initiated on each shoot.

In order to detect the fruit set percentage and fruit abscission, four branches with almost equal buds in different geographical sides of each tree were selected and marked. These factors were measured by the following formula, respectively:

$$\text{Initial fruit set percentage} = \frac{\text{Numer of fruit set (30th day)}}{\text{Number of flower buds}} \times 100$$

$$\text{Harvest fruit set percentage} = \frac{\text{Numer of fruit set (120th day)}}{\text{Number of flower buds}} \times 100$$

*Fruit characteristics*

At the harvest time, all clusters were detached from each shoot and were hand-sorted into blank, non-split, and split nuts, fresh and dry weight of fruits were measured on each marked branches. Yield factor is reported in results as dry weight of nuts per each branch.

*Biochemical characteristics*

All biochemical parameters were measured about two weeks after foliar application of brassinosteroides and spectrophotometer was used for all biochemical measurement.

*Assay of protein, APX and POD*

Fresh leaf samples (1 g) from all treatments were ground in 5 ml Tris-HCl buffer (0.05 M). The homogenates were centrifuged at 10,000 *g* for 25 min at 4°C. The protein content in the supernatant was analyzed by the procedure of Bradford (1979). The supernatants were also used to analyze the activity of APX and POD enzymes. APX activity was determined by Nakano and Asada method (1981).

*Assay of SOD, CAT and $H_2O_2$*

Frozen leaf samples (1 g) were homogenized in 50 mM sodium phosphate buffer (pH 7.8 for SOD and pH 7 for CAT). Then, these materials were centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was used to measure the activity of the enzymes.

SOD activity was measured according to the method described by Giannopolitis and Ries (1977) and CAT activity was determined by the procedure of Cakmak and Marschner (1992).

*Reducing sugars and Proline analysis*

Proline and reducing sugars content were extracted from the fresh leaf samples according to Bates *et al*. (1973) and Somogyi (1952), respectively.

*Chlorophyll content of leaves*

The Chlorophyll content was calculated using the method introduced by Meidner (1984) as:

$$\text{Total Chlorophyll (mg/ml}^{-1}\text{ g}^{-1}\text{): } [(17.76 \times OD646.6) + (7.37 \times OD663.6)] \times V/1000W$$

where, OD= the read absorbance , V= consumed acetone volume, W= fresh weight of sample (g).

*Ion leakage of leaves*

The relative permeability of cell membranes was calculated using a slight modification of the method introduced by Zhang *et al.* (2006) as:

$$\text{Relative permeability (\%)} = \frac{EC_1 - EC_0}{EC_2 - EC_0} \times 100$$

*Total nonstructural carbohydrate of 1-year old wood*

One-year-old stems were immediately placed on ice and were taken to the laboratory. In the laboratory, the stem tissue were dried at 60°C, weighted, ground to pass a 40-mesh screen and analyzed for total nonstructural carbohydrate (TCN). The concentration of starch was measured by the method of Hedge and Hofreiter (1962). Dissolved sugar was calculated using the phenol and sulphuric acid method introduced by Hellebust and Craige (1978). The absorbencies were measured at 485 and 630 nm for dissolved sugar and starch, respectively. The concentration of total sugar and starch were summed to

give an estimate of total nonstructural carbohydrate.

*Statistical analysis*

Finally, the collected data were analysed using SAS software package, the means were compared by Duncan test at 5% level, and the diagrams were drawn in MS-Excel software package.

## 3. Results

*Interaction of brassinosteroides and cultivars for inflorescence bud, fruit abscission, and fruit set in pistachio*

The result indicated that the two-stage application of this hormone decreased bud abscission by about 20% in Kalleghuchi cultivar but by about 10% in other cultivars (Akbari and Ohadi) (Table 2). In the "on" year, the highest percentage of fruit abscission was observed in control in Kalleghuchi cultivar and the application of this hormone especially in two stages decreased fruit abscission by about 30% while fruit abscission was decreased by 20% and 18% in Akbari and Ohadi cultivars, respectively (Table 2) and also the foliar spray of this hormone in two stages increased the fruit set significantly so that it was doubled as compared to control.

*Interaction of brassinosteroides application time and cultivars for blank and split nuts in Pistachio*

The highest blank nut and the lowest split nut were observed in control in 'Kallehghoochi' in two experimental years and in the "on" year, they were about 6% and 54%, respectively. The lowest blank nut and the highest split nut were observed in Kallehghoochi and Ohadi cultivars and brassinosteroides spray at two stages in the "on" year at about 4% and 80%, respectively. The percentage of blank nut was decreased in all cultivars in the "off" year by about 50% when the hormone was applied at two stages and by 25% when it was applied at one stage. In the "off" year, the percentage of split nut was significantly increased only in 'Kallehghoochi' at two stages of brassinosteroides application (Table 3).

Table 2 - Interaction of stage of brassinosteroides application and cultivar for fruit and bud abscission and fruit set percentage of pistachio in two years

| | 'Ohadi' | | | | 'Akbari' | | | | 'Kallehghoochi' | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | C | T1 | T2 | T3 | C | T1 | T2 | T3 |
| *During 2014 "on year"* | | | | | | | | | | | | |
| Fruit set (%) | 9.5 d | 13 c | 14 c | 16 bc | 11 d | 14.5 c | 18 b | 21 a | 7.1 f | 8.5 e | 9.2 d | 13.5 c |
| Friut abscission (%) | 58 b | 50 c | 47 c | 40 d | 62 ab | 54 bc | 55 b | 42 C | 68 a | 47 c | 43 c | 36 d |
| Bud abscission (%) | 68 c | 63 d | 60 d | 58 d | 88 a | 83 b | 80 b | 78 b | 90 a | 78 b | 72 c | 68 c |
| *During 2015 "off year"* | | | | | | | | | | | | |
| Fruit set (%) | 11 d | 12.5 c | 13 c | 14 c | 15 b | 16 b | 17 b | 19 a | 9 d | 9.5 d | 10 d | 12 c |
| Fruit abscission (%) | 44 c | 42.5 cd | 40 d | 38 d | 50 b | 46 c | 47 bc | 42 cd | 54 a | 50 b | 48 bc | 45 c |
| Bud abscission (%) | 50 a | 45 ab | 43 ab | 40 b | 30 c | 28 c | 28 c | 22 d | 25 cd | 24 cd | 22 d | 22 d |

C= Control, T1= Bud swelling phase, T2= Two weeks after full bloom, T3= T1 and T2.
Different letters within a row indicate significant differences by Duncan's multiple range tests at P<0.05.

Table 3 - Interaction of stage of brassinostroides application and cultivar for blank, split, fresh and dry weight of nut, and yield per shoot of pistachio in two years

| | 'Ohadi' | | | | 'Akbari' | | | | 'Kallehghoochi' | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | C | T1 | T2 | T3 | C | T1 | T2 | T3 |
| *During 2014 "on" year* | | | | | | | | | | | | |
| Blank nut (%) | 4.5 bc | 4.2 c | 4 c | 3.2 d | 5.5 ab | 5 b | 4.5 bc | 4 c | 6 a | 5 b | 4.5 bc | 4 c |
| Fresh weight nut (g) | 2.1 e | 2.1 e | 2.4 d | 2.9 c | 2.9 c | 3 c | 3.2 b | 3.8 a | 2.9 c | 3 c | 3.2 b | 3.9 a |
| Dry weight nut (g) | 0.67 d | 0.7 d | 0.8 cd | 0.95 c | 0.9 c | 1 bc | 1.1 b | 1.2 ab | 0.9 c | 1 bc | 1.1 b | 1.3 a |
| Split nut (%) | 50 d | 70 b | 65 bc | 75 ab | 65 bc | 70 b | 75 ab | 79 a | 54 d | 63 c | 70 b | 80 a |
| Yield per shoot (g) | 45 e | 75 c | 80 c | 100 b | 60 d | 90 b | 95 c | 120 a | 50 ed | 65 d | 75 c | 90 bc |
| Yield per tree (kg) | 2 f | 2.5 ef | 3 e | 3.5 de | 4 d | 5 c | 6.1 b | 7 a | 3 e | 3 e | 4.1 d | 5.2 c |
| *During 2015 "off" year* | | | | | | | | | | | | |
| Blank nut (%) | 10 b | 8.5 c | 8 c | 5.5 d | 12 a | 8.5 c | 9 bc | 6d | 11 ab | 7 cd | 6.5 d | 6 d |
| Fresh weight nut (g) | 2.8 d | 3 cd | 3 cd | 3.8 a | 3.2 c | 3.7 ab | 3.6 b | 3.9 a | 3.3 c | 3.5 b | 3.5 b | 3.9 a |
| Dry weight nut (g) | 0.9 e | 1 d | 1 d | 1.2 b | 1.1 c | 1.2 b | 1.2b | 1.3 a | 1.1 c | 1.2 b | 1.2 b | 1.3 a |
| Split nut (%) | 75 bc | 76 bc | 78 b | 80 b | 8 ab | 87 a | 87 a | 90 a | 73 c | 80 b | 80 b | 80 b |
| Yield per shoot (g) | 14 e | 20 b | 18 c | 22 a | 15 ed | 20 b | 20 b | 22 a | 16 d | 20 b | 18 c | 22.1 a |
| Yield per tree (kg) | 1 d | 1.1d | 1.5 c | 1.8 b | 1.5 c | 1.8 b | 2.1 b | 2.8 a | 1.3 c | 1.3 c | 1.7 bc | 2 b |

C= Control, T1= Bud swelling phase, T2= Two weeks after full bloom, T3= T1 and T2.
Different letters within a row indicate significant differences by Duncan's multiple range tests at P<0.05.

*Interaction of brassinosteroides application time and cultivars for fresh and dry weight of pistachio nut*

The highest fresh weight and dry weight of fruit were observed in 'Kallehghoochi' and in brassinosteroides spray at two stages, which were about 3.9 and 1.3 g, respectively. The lowest fresh and dry weights of fruit in the "on" year were observed in control in 'Ohadi' with no hormone application, which were about 2.1 and 0.67 g, respectively (Table 3).

*Interaction of brassinosteroides application time and cultivars for yield per shoot and tree in pistachio*

Table 3 showed that brassinosteroides significantly increased the yield per shoot and tree, especially when they were applied at two stages in the "on" year. The interaction between cultivar and the stage of brassinosteroides spray for the yield per shoot showed that the highest yield of branch and tree were observed in 'Akbari' and in brassinosteroides spray at two stages, which were about 120 g and 7 kg, respectively. The lowest yield of branch and tree were observed in control of Ohadi cultivar and they were about 45 g and 2 kg, respectively in the "on" year (Table 3).

*Interaction of brassinosteroides application time and cultivars on proline, reduced sugar, and protein of pistachio leaves*

Table 4 reveals that the highest amount of reduced sugar and the lowest amount of protein and proline was observed in control in 'Kallehghuchi' in both experimental years. The application of brassinosteroides (at two stages) increased protein and proline contents of the leaves in all pistachio cultivars significantly, especially in the "on" year, and also the lowest amount of reduced sugar was observed in all cultivars in both "on" and "off" years when the hormone was applied at two stages (Table 4).

*Interaction of the time of brassinosteroides application and cultivars for chlorophyll and ion linkage of leaves*

According to Table 4, the chlorophyll content was significantly increased in all brassinosteroides treatments as compared to control. Maximum chlorophyll content was observed in two-stage application of this hormone in all cultivars. Maximum ion leakage was related to the control in all cultivars and all brassinosteroides treatments showed significantly lower ion leakages. Minimum ion leakage was observed in two-stage application of this hormone in all cultivars (Table 4).

*Interaction of the time of brassinosteroides application and cultivars for antioxidant enzyme activities*

Figures 1 and 2 shows that activity of all antioxidant enzymes was significantly higher in the "on" year than in the "off" year. The application of brassinosteroides suppressed the activity of all enzymes (CAT, SOD and APX) except POD. The lowest activity of all enzymes was observed in control of Kallehghuchi cultivar in two trial years so that they were remarkably boosted with application of this hormone as compared to other cultivars. Maximum enzyme activity was observed in two-stage application of this hormone in all cultivars.

*Interaction of the time of brassinosteroides application and cultivars on annual changes in total non-structural carbohydrate (TCN) concentration of one-year-old wood of pistachio*

Figures 3 and 4 reveal that carbohydrate storage

Table 4 - Interaction of stage of brassinostroides application and cultivar for some physiological traits of pistachio in two years

| | 'Ohadi' | | | | 'Akbari' | | | | 'Kallehghuchi' | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | C | T1 | T2 | T3 | C | T1 | T2 | T3 |
| *During 2014 "on" year* | | | | | | | | | | | | |
| Proline (µm/g FW) | 20 c | 22.6 c | 25.7 bc | 35 ab | 20 c | 23 c | 28 b | 38 a | 18 c | 20 c | 30 b | 40 a |
| Reducing sugars (mg/g FW) | 30 b | 25 bc | 24 bc | 18 c | 35 b | 30 b | 26 bc | 20 c | 40 a | 30 b | 25 bc | 12 d |
| Protein (mg/g FW) | 18 bc | 19.1 b | 20 ab | 21 a | 19 b | 19 b | 19 b | 21 a | 15 d | 16.8 c | 19 b | 2 ab |
| Ion Leakage (%) | 38 b | 34 c | 35 bc | 31 cd | 34 c | 30 d | 30 d | 29 d | 43 a | 40 ab | 39 b | 34 c |
| Chlorophyll (mg/ml FW) | 21 c | 25 b | 28 ab | 29.9 a | 19.5 c | 26 b | 27 ab | 30.3 a | 16.3 d | 26.5 b | 27.3 ab | 30.2 a |
| *During 2015 "off" year* | | | | | | | | | | | | |
| Proline (µm/g FW) | 15 c | 16 c | 16.2 c | 20 b | 14.5 c | 14.5 c | 15c | 19 b | 14.2 c | 17.5 bc | 20.5 b | 25.8 a |
| Reducing sugars (mg/g FW) | 7.7 ab | 7.56 b | 7.5 b | 6.9 c | 6.5 d | 6.5 d | 6 e | 6 e | 7. 9 a | 7.75 ab | 7.75 ab | 7.42 b |
| Protein (mg/g FW) | 17.1 bc | 17.1 bc | 17.5 b | 17.7 b | 19 ab | 19 ab | 19.1 ab | 20.1 a | 16 c | 16.5 c | 18.3 b | 20 a |
| Ion Leakage (%) | 40 b | 37 c | 37 c | 32 d | 36 c | 33 d | 31 d | 29 e | 45 a | 43 ab | 41 b | 38 bc |
| Chlorophyll (mg/ml FW) | 25 c | 28 bc | 32 ab | 34 a | 22 d | 29 b | 30 b | 33 a | 20 d | 29 b | 30 b | 33 a |

C= Control, T1= Bud swelling phase, T2= Two weeks after full bloom, T3= T1 and T2.
Different letters within a row indicate significant differences by Duncan's multiple range tests at P<0.05.

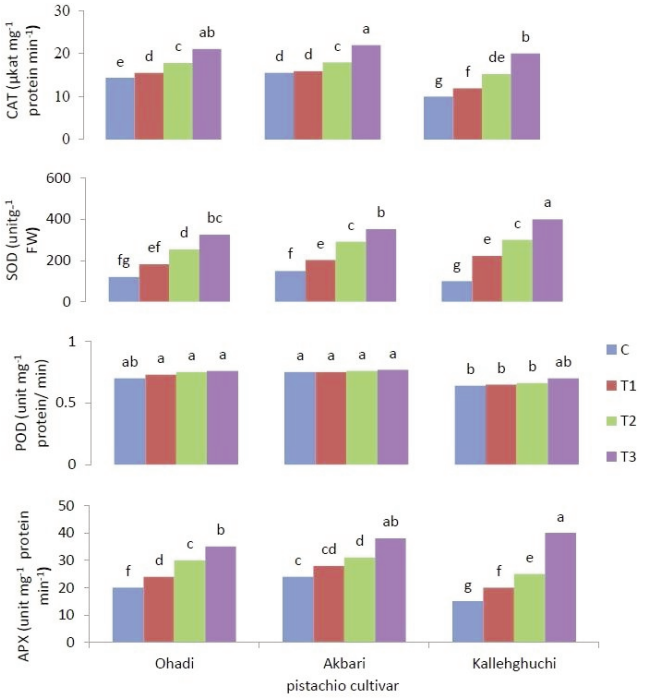



Fig. 1 - Interaction of stage of brassinostroides application and cultivar for the activities of some antioxidant enzyme of pistachio in the "on" year. C= Control, T1= Bud swelling phase, T2= two weeks after full bloom, T3= T1 and T2. Different letters within a row indicate significant differences by Duncan's multiple range tests at P<0.05.

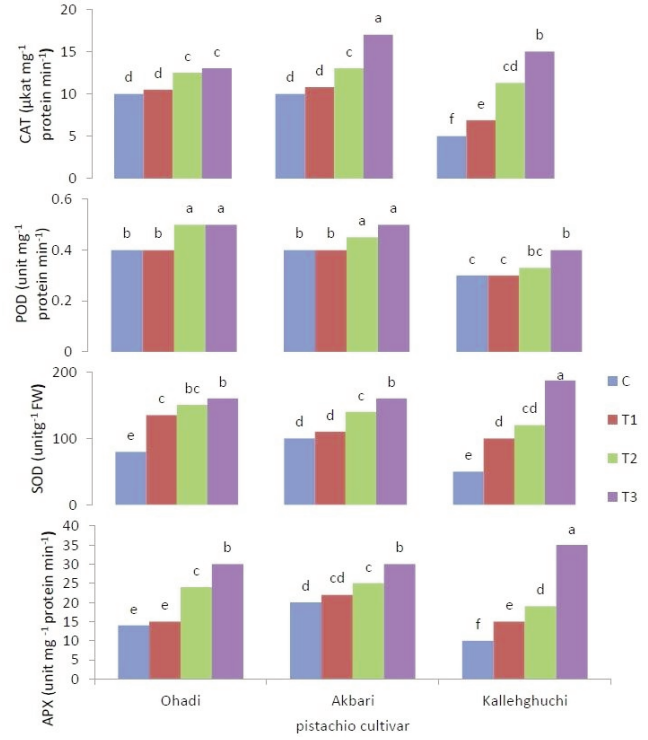



Fig. 2 - Interaction of stage of brassinostroides application and cultivar on activities of some antioxidant enzyme of pistachio in the "off" year. C= Control, T1= Bud swelling phase, T2= two weeks after full bloom, T3= T1 and T2. Different letters within a row indicate significant differences by Duncan's multiple range tests at P<0.05.

in shoots of "on" and "off" trees decreased following the spring growth flash. Figure 3 shows that, in "on" trees, the lowest amount of carbohydrate was observed in Jun and Aug in all cultivars. After harvest (Sep), amount of carbohydrate increased again. Figure 4 shows that, in "off" trees, stored carbohy-

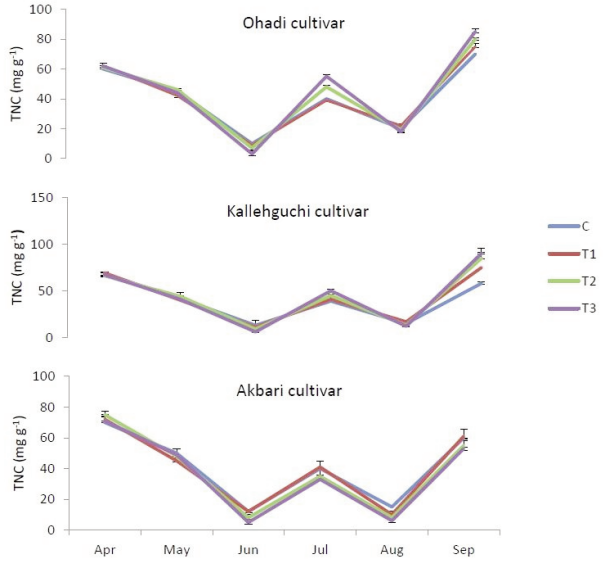



Fig. 3 - Interaction of stage of brassinostroides application and cultivar on annual changes in total non-structural carbohydrate (TCN) concentration of one-year-old wood of pistachio in the "on" year. C= Control, T1= Bud swelling phase, T2= two weeks after full bloom, T3= T1 and T2.

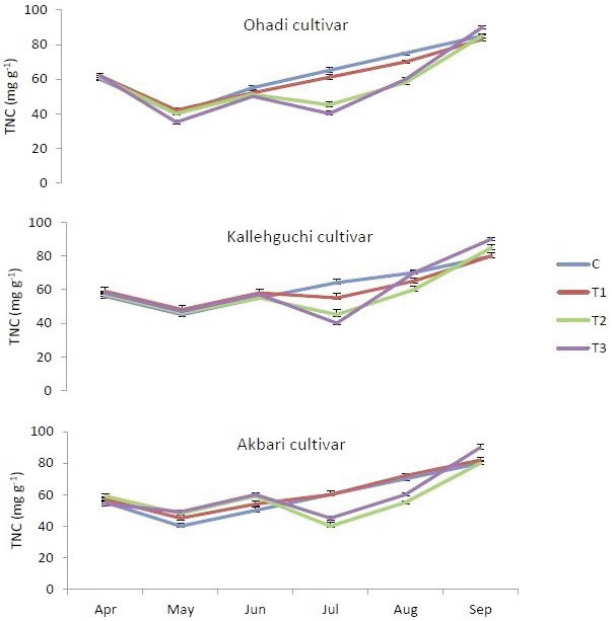



Fig. 4 - Interaction of stage of brassinostroides application and cultivar on annual changes in total non-structural carbohydrate (TCN) concentration of one-year-old wood of pistachio in the "off" year. C= Control, T1= Bud swelling phase, T2= two weeks after full bloom, T3= T1 and T2.

drate of control and T1 treatments increased and remained high after initial growth flash but, in T3 and T4 treatments, it decreased in July and then started to increase again.

**4. Discussion and Conclusions**

The effect of 24-epibrassinolid application on yield and quantitative traits of pistachio in three cultivars was considerable in this experiment. Maximum yield was observed in Akbari cultivar and brassinosteroides spray at two stages so that it was doubled as compared to the control. Also, the application of this hormone at two stages increased fruit set significantly as compared to control. This finding confirms those reported by Heidari *et al.* (2015) who showed that the application of epi-brassinolid increased fruit set in Alberta peach. Brassinosteroides regulate fruit number and are also able to induce cell division and elongation (Rao *et al.*, 2002). Gomes *et al.* (2006) reported that the use of Brassinosteroides in yellow passion after flowering has led to an increase in fruit number and yield. Foliar application of brassinosteroids resulted in an increase in the number of flowers in strawberry (Leubner-Metzger, 2001), and also in grape fruits, the foliar application of brassinosteroids in autumn increased the number of flowers (Pipattanawong *et al.*, 1996). These results show that the increased yield in this experiment could be due to the increase in pistachio flowers induced by this hormone.

Another problem in pistachio is fruit abscission whose causes are not clearly known. However, environmental influences and competition for resources such as photo assimilates and plant hormones are the likely causal factors of fruit abscission (Thompson, 1996). fruit abscission in this experiment fell by half in Kallehghuchi cultivar at two-stage hormone application. Foliar spray of epibrassinolid on grape (0.01 mg/l) during flowering reduced fruit abscission (Pozo *et al.*, 1994). Brassinosteroides can increase carbohydrate content because of the increase in photosynthesis capacity and translate assimilate from source to sink (Goetz *et al.*, 2000).

Evaluation of the carbohydrate storage in one-year-old wood of pistachio in spring and summer season in this research showed that the amount of carbohydrate decreased in spring (April and May).

This reduction of stored carbohydrate could be related to spring growth flash. In “on” year, there were other two steps of reduction of carbohydrate, the first and second steps were due to summer growth flash and period of kernel fill, respectively. This finding confirms the results of Timothy *et al*. (2008). But it should be noted that the application of this hormone, especially in T3 treatment, decreased significantly the amount of stored carbohydrates in first step of reduction as compared to control. This seems to be due to stimulation of growth after application of this hormone. Stored carbohydrates, in one-year-old wood, after harvest of pistachio were significantly higher in this treatment. In “off” trees, stored carbohydrate of control and T1 treatments increased and remained high after initial growth flash but stored carbohydrate in T3 and T4 treatments decreased in July and then started to increase again. It seems that decreased carbohydrate in July could be caused by the stimulation of growth by the use of hormone. Thus, other reason for higher yield in this experiment is the enhanced growth after application of this hormone caused by the increased number of leaves and shoot and, consequently, more assimilation synthesis for kernel growth and flower bud development.

Chlorophyll content of leaves was increased in the treated plants. Thus, the increased chlorophyll could be the reason for the increase in assimilates in pistachio, resulting in the increased fruit set, fresh and dry weight of nuts and yield. This is consistent with the results of Hassan Zadeh (2013) who showed that the application of epi-brassinolid increased chlorophyll in cantelop.

Unfavourable environmental stresses such as drought and high temperature conditions during flowering and after it decrease fruit set and, on the other hand, increase fruit abscission in most pistachio gardens in Rafsanjan. Plant stress tolerance requires the activation of complex metabolic activities including anti-oxidative pathways, especially ROS-scavenging systems within the cells that in turn can contribute to continued plant growth under stress conditions (El-Mashad and Mohamed, 2012). One of the roles of Brassinosteroids is the increased resistance of plants against various abiotic stresses as confirmed by the results of this experiment. So, APX, SOD and CAT enzymes were increased with the application of this hormone especially its two-stage application. It should also be noted that in ‘Kallehghuchi’ as the most sensitive cultivar to physiological disorder, the amount of antioxidant enzymes were lower than other cultivars and that it was increased with the application of brassinosteroides hormone significantly. There are numerous reports in this regard,

some of which are reviewed here. Brassinosteroid-treated tomato and rice plants grew better than control plants under low-temperature conditions (Kamuro and Takatsuto, 1991). In rice, 24-epibrassinolide increased the resistance against chilling stress (1-5°C) and the tolerance was associated with the increased ATP, proline levels and SOD activity, thus indicating brassinosteroid involvement in membrane stability and osmo regulation (Wang and Zang, 1993). Brassinosteroids increased tolerance to high temperature in wheat leaves (Kulaeva *et al.*, 1991).

Results of this experiment showed that the reduction of ion leakage in leaves confirms that this hormone impacts membrane permeability, resulting in resistance to environmental stress. Thus, the increased fruit set and decreased fruit abscission in treated trees could be due to the increased resistance to a biotic stress because of hormone application. On the other hand, the application of epi-prassinolid increased proline content and decreased the reduced sugar in pistachio leaves. It seems 24-epi brassinolid could alleviate the adverse effects of abiotic stress on pistachio cultivars, especially on Kallehghuchi arguably by increasing the activities of ant oxidative enzymes and the contents of proline. The increase in proline and anti-oxidant enzymes are the natural mechanisms to induce tolerance to abiotic stress in plants. In a similar study, Rady (2011) reported that the spray of 5µM 24-epi brassinosteroides to NaCl-exposed *Phaseolus vulgaris* improved the elevation of the activities of anti-oxidative enzymes and proline content. Aghdam *et al.* (2012) reported that the treatments with 0.3 and 0.6 µM BRs to tomato fruits stored at 1°C for 21 days enhanced proline. Foliar application of brassinosteroides was reported to improve Cd-tolerance in *Brassica juncea* through the increase in activity of antioxidative enzymes (such as CAT, POD, SOD) and the content of osmolyte such as proline (Hayat *et al.,* 2007). On the other hand, lower amount of reduced sugar in brassinosteroides treatments could reflect the alleviation of the adverse effects of abiotic stress after the application of this hormone.

Dry and fresh weight of pistachio nuts was increased by about 30% with the application of 24-epibrassinolid at two stages. The increase of nut weight may be another reason for the increased yield in this experiment. Brassinosteroides have important role in such phenomenon as cell division and elongation, photosynthesis rate, transfer material, regulation of enzyme activity and hormone balance, each of which can increase nut weight and yield in pistachio crop. Increased DNA and RNA polymerase and increased synthesis of protein by the use of Brassinosteroides can induce growth (Kalinich *et al.*, 1985). Vardhini and Rao (1998) showed the relationship between increased growth and DNA, RNA and protein synthesis in peanut. Yu (1999) found significant increases in the initial activity of Rubisco and in the sucrose, soluble sugars, and starch contents followed by substantial increases in sucrose phosphate synthase, sucrose synthase and acid invertase activities after BRs treatment. Impaired carbohydrate metabolism and reduced biomass were found in a brassinosteroid-deficient *Arabidopsis* mutant (Schluter *et al.*, 2002).

Increasing of blank nut especially in Kallehghoochi and Akbari cultivars is one of the most important disorders in pistachio trees. It has been reported that the degeneration of the ovary segments, especially funicle degeneration, is the major cause of blanking in pistachio (Shuraki and Sedgley, 1996). Brassinosteroids are considered as hormones with pleiotropic effects as they influence various developmental processes like growth, flowering, pollen tube growth, tissue differentiation, proton pump activation, gene expression and photosynthesis (Khripach *et al.*, 1998, 2000). Therefore, brassinosteroide can improve growth and development of reproductive organs and prevent ovary degeneration and, on the other hand, embryo abortion could be decreased by the application of this hormone because of the inducing protein, carbohydrate synthesis and, consequently, the blank nuts can be decreased.

In this research the percentage of non-split nuts was decreased with the application of epibrassinolid. A correlation has been found between kernel development and splitting (Ferguson *et al.*, 2005). Thus, the decrease in the percentage of non-split nuts by the application of this hormone might be attributed to its role in improving the growth and development of pistachio trees and, on the other hand, the application of epibrassinolid increases the production of carbohydrate and assimilation into the plants and, as a result, the growth of kernel increases (Crane and Iwakiri, 1985).

In conclusion, exogenous application of 24-epibrassinolide indicated the possible direct role of this hormone in alleviating the physiological disorders and increasing the quality and yield in pistachio. Thus, 24-epibrassinolide is a good suggestion for increasing quantity and quality of pistachio, especially in Kallehghoochi cultivar that has the most disorder physiological problems.

## References


AGHDAM M.S., ASGHARI M., FARMANI B., MOHAYEJI M., MORADBEYGI H., 2012 - *Impact of postharvest brassinosteroids treatment on PAL activity in tomato fruit in response to chilling stress.* - Horticultural Science, 144: 116-120.

ANJUM N.A., UMAR S., AHMAD A., 2012 - *Oxidative stress in plants: Causes, consequences and tolerance.* - First ed., IK International Publishing House, New Delhi, India, pp. 543.

ANJUM N.A., UMAR S., CHAN M.T., 2010 - *Ascorbate-glutathione pathway and stress tolerance in plants.* - First ed., Springer, Dordrecht, The Netherlands, pp. 545.

BATES L., WALDREN P.P., TEARE J.D., 1973 - *Rapid determination of the free proline of water stress studies.* - Plant and Soil Journal, 39: 205-207.

BRADFORD M.N., 1979 - *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding.* - Annual Review of Biochemistry, 72: 248-254.

CAKMAK I., MARSCHNER H., 1992 - *Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves.* - Plant Physiology, 98: 1222-1227.

CLOUSE S.D., SASSE J.M., 1998 - *Brassinosteroids: essential regulators of plant growth and development.* - Annual Review of Plant Physiology and Plant Molecular Biology, 49: 427-451.

CRANE J.C., IWAKIRI B.T., 1985 - *Vegetative and reproductive dominance in pistachio.* - Horticultural Science, 20: 1092-1093.

DHAUBHADEL S., CHAUDHARY S., DOBINSON.K.F., KRISHNA P., 1999 - *Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermo tolerance of* Brassica napus *and tomato seedlings.* - Plant Molecular Biology, 40: 333-342.

EL-MASHAD A., MOHAMED H., 2012 - *Brassinolide alleviates salt stress and increases antioxidant activity of cow pea plants (*Vigna sinensis*).* - Protoplasma, 249: 625-635.

FERGUSON L., BEEDE R.H., FREEMAN M.W., HAVILAND D.R., HOLTZ B.A., KALLSEN C.E., 2005 - *Pistachio production manual.* - Fourth ed. Fruit and Nut Research and Information Center, University of California, Davis, California, USA, pp. 251.

GIANNOPOLITIS C.N., RIES S.K., 1977 - *Superoxide dismutases. I. Occurrence in higher plants.* - Plant Physiology, 59: 309-314.

GOETZ M., GODT D.E., OITSCH T.R., 2000 - *Tissue-specific induction of the mRNA for an extracellular invertase isoenzyme of tomato by brassinosteroids suggests a role for steroid hormones in assimilate partitioning.* - Plant Journal, 22: 515-522.

GOMES M., CAMPOSTRIN E., EAL N.R.L., 2006 - *Brassinosteroid analogue effects on the yield of yellow passion fruit plants (*Passiflora edulis*).* - Scientia Horticulturae, 110: 235-240.

HASSAN ZADEH S.H., 2013 - *Evaluation effect of epibrassinolid on vegetative growth, yield and quality traits of cantaloupe (*Cucumis melon *l.).* - M.Sc. thesis, Bahonar University, Kerman, Iran.

HAYAT S., ALI B., HASAN S.A., AHMAD A., 2007 - *Brassinosteroid enhanced the level of antioxidants under cadmium stress in* Brassica juncea*.* - Environmental and Experimental Botany, 60: 33-41.

HEDGE J.E., HOFREITER B.T., 1962 - *Estimation of starch by anthrone reagent.* - In: WHISTLER R.L., and J.N. BEMILLER (eds.) *Methods in carbohydrate chemistry*. *Analysis and preparation of sugars*. Academic Press, New York, USA, pp. 580.

HEIDARI T., ARVIN M.J., TAVASOLIAN I., 2015 - *Impact of 24-Epibrassinolide on some quantitative of peach fruit cv. 'Alberta'.* - J. Sci. Techn., 15(3): 399-406.

HELLEBUST J.A., CRAIGE J.S., 1978 - *Handbook of physiological methods. Physiological and biochemical methods.* - Cambridge University Press, London, UK, pp. 430.

HOSSEINPUR M.R., AKBARNASAB A., PAKKISH Z., 2014 - *Effect of brassinosteroides on reproductive growth of Kallehghuchi pistachio.* - First symposium of pistachio, 31 July, Bahonar University, Kerman, Iran.

KALINICH J.F., MANDAVA N.B., TODHUNTER J.A., 1985 - *Relationship of nucleic acid metabolism to brassinolide induced responses in beans.* - Plant Physiology, 120: 207-214.

KAMURO Y., TAKATSUTO S., 1991 - *Capability for problems of practical uses for brassinolides,* pp. 292-297. - In: CUSTLER H.G., T. YOKOTA, and G. ADAM (eds.) *Brassinosteroids. Chemistry, bioactivity and application. ACS Symposium Series*. Am. Chem. Soc., Washington, USA.

KHRIPACH V., ZHABINSKII V.., DE GROOT A., 2000 - *Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for XXI century*. - Annals of Botany, 86(3): 441-447.

KHRIPACH V.A., ZHABINSKII V.N., DE GROOT A.E., 1998 - *Brassinosteroids: A new class of plant hormones.* - Academic Press, San Diego, CA, USA, pp. 456.

KRISHNA P., 2003 - *Brassinosteroids-mediated stress responses.* - Plant Growth Regulation, 22: 289-297.

KULAEVA O.N., BURKHANOVA E.A., FEDINA A.B.V., 1991 - *Effects of brassinosteroides on protein synthesis and plant cell ultrastructure under stress condition,* pp. 141-155*.* - In: CUTLER H.G., T. YOKOTA, and G. ADAM (eds.) *Brassinosteroids. Chemistry, bioactivity and applications*. Amer. Chem. Soc., Washington, USA, pp. 358.

LEUBNER-METZGER G., 2001 - *Brassinoides and Giberlines promote tobacco seed germination*. - Planta, 213: 758-763.

MANDAVA N.B., 1988 - *Plant growth-promoting brassinosteroids*. - Annual Review of Plant Physiology and Plant Molecular Biology, 39: 23-52.

MEIDNER H., 1984 - *Class experiments in plant physiology*.

- British Library Cataloguing in Publication Data, Oxford University press, London, UK.

NAKANO Y., ASADA K., 1981 - *Hydrogen peroxide is scavenged by ascorbate-peroxidase in spinach chloroplast.* - Plant and Cell Physiology, 22: 867-880.

PIPATTANAWONG N., FUJISHIGE N., YAMANE K., OGATA R., 1996 - *Effect of brassinosteroid on vegetative and reproductive growth in two day-neutral strawberries.* - J. Jpn. Soc. Hortic. Sci., 65: 651-654.

POZO L., NORIEGA C., ROBAINA C., COLL F., 1994 - *Foliar spraying of epibrassinolid on grape (*Vitis vinifera*).* - Cult. Trop., 15: 79-92.

RADY M.M., 2011 - *Effect of 24-epibrassinolide on growth, yield, antioxidant system and cadmium content of bean (*Phaseolus vulgaris *L.) plants under salinity and cadmium stress.* - Scientia Horticulturae, 129: 232-237.

RAMRAJ V.M., VYAS B.N., GODREJ N.B., MISTRY K.B., SWAMY B.N., SINGH N., 1997 - *Effects of 28-homobrassinolide on yields of wheat, rice, groundnut, mustard potato and cotton.* - J. Agric. Sci., 128: 405-413.

RAO S.S.R., VARDHINI B.V., SUJATHA E., ANURADHA S., 2002 - *Brassinosteroids - A new class of phytohormones.* - Current Science, 82(10): 1239-1245.

SCHLUTER U., KOPKE D., ALTMANN T., MUSSING C., 2002 - *Analysis of carbo-hydrate metabolism of CPD antisense plants and the brassino-steroid-deficient cbbl mutant.* - Plant Cell Environ., 25: 783-791.

SHURAKI Y.D., SEDGLEY M., 1996 - *Fruit development of* Pistacia vera *(*Anacardiaceae*) in relation to embryo abortion and abnormalities at maturity.* - Australian Journal of Botany, 44: 35-45.

SOMOGYI M., 1952 - *Notes on sugar determination.* - J. Biol. Chem., 195: 19-29.

STEBER C.M., McCOURT P., 2001 - *A role for brassinosteroids in germination in* Arabidopsis. - Plant Physiology, 125: 763–769.

SYMONS G.M., DAVIES C., SHAVRUKOV Y., DRY I B., REID J., THOMAS M.R., 2006 - *Grapes on steroids. Brassinosteroids are involved in grape berry ripening.* - Plant Physiology, 140: 150-158.

TAKATSUTO S., YAZAWA N., IKEKAWA N., TAKEMATSU T., TAKEUCHU Y., KOGUCHI M., 1983 - *Structure-activity relationship of brassinosteroids*. - Phytochemistry, 22: 2437-2441.

THOMPSON M., 1996 - *Flowering, pollination and fruit set*, pp. 223-241. - In: WEBSTER A.D., and N.E. LOONEY (eds.) *Cherries: Crop physiology, production and uses*. CABI, Wallingford, Oxfordshire, UK, pp. 464.

TIMOTHY M., ROBERT H., DEJONG M., 2008 - *Seasonal carbohydrate storage and mobilization in bearing and non-bearing of pistachio.* - Tree Physiology, 28: 207-213.

VARDHINI B.V., RAO S.S., 2002 - *Acceleration of ripening of tomato pericarp discs by brassinosteroids*. - Phytochemistry, 61: 843-847.

VARDHINI B.V., RAO S.S.R., 1998 - *Effect of brassinosteroids on growth, metabolite content and yield of* Arachis hypogaea*.* - Phytochemistry, 48: 927-930.

VERT G., NEMHAUSER J.L., GELDNER N., HONG F.X., CHORY J., 2006 - *Molecular mechanisms of steroid hormone signalling in plants.* - Annual Review of Cell and Developmental Biology, 21: 177-201.

WANG B.K., ZANG G.W., 1993 - *Effect of epibrassinolide on the resistance of rice seedlings to chilling injury*. - Acta Physiologica, 19: 38-42.

YU J.Q., HUANG L.F., HU W.H., ZHOU Y.H., MAO W.H., YE S.F., NOGUES S., 2004 - *A role for brassinosteroids in the regulation of photosynthesis in* Cucumis sativus*.* - J. Exper. Bot., 55: 1135-1143.

YU J.Q., 1999 - *Parthenocarpy induced by N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) prevents flower abortion in Chinese white-flowered gourd (*Lagenaria leucantha*).* - Environ. Exper. Bot., 42: 121-128.

ZHANG J., LIU Y.P., PAN Q.H., ZHAN J.C., WANG X.Q., HUANG W.D., 2006 - *Changes in membrane-associated $H^+$-ATPase activities and amounts in young grape plants during the cross adaptation to temperature stresses.* - Plant Sci., 170: 768-777.

AHS
Advances in Horticultural Science

# Storage conditions of soft X-ray irradiated pollen for producing seedless watermelons

**M. Akutsu**[1, 2 (*)]

[1] *National Agricultural Research Center for Hokkaido Region, Hokkaido, Japan.*

[2] *Department of Electrical Engineering and Computer Science, Tokai University, Kumamoto, Japan.*

*Key words*: Fruit quality, $N_2$ storage, seedless watermelon, soft x-ray, storage temperature, vacuum storage.



(*) **Corresponding author:**
akutsu@tsc.u-tokai.ac.jp

**Citation:**
AKUTSU M., 2018 - *Storage conditions of soft X-ray irradiated pollen for producing seedless watermelons.* - Adv. Hort. Sci., 32(1): 13-17



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 12 September 2017
Accepted for publication 8 November 2017

**Abstract: A safe and efficient method for preserving viable soft X-ray-irradiated pollen for the production of seedless watermelon (*Citrullus lanatus* L.) from diploid plants was tested by packing the pollen under vacuum, $O_2$, $CO_2$ or $N_2$ gas at 25°C, 4°C or -25°C. Pollen germination rates decreased most rapidly with storage at 25°C and slowest with storage at -25°C. Oxygen as a storage gas was not good for storage of pollen, but pollen stored in $N_2$ or $CO_2$ gave good germination. Pollen stored at -25°C for 90 days germinated, but pollen stored at 4°C for 90 days did not, and $N_2$ storage tended to result in higher fruit set than vacuum storage. Fruit set was significantly affected by pollen storage conditions, with $N_2$ storage being more effective than vacuum storage at 4°C. Storage at -25°C produced little difference in fruit set between vacuum and $N_2$ storage. Thus, temperature was the major factor for maintaining viable and effective pollen, and the use of $N_2$ gas was an effective adjunct. Fruit quality was not significantly affected by storage parameters in this experiment.**

## 1. Introduction

Generally, seedless watermelons are produced from triploid plants that are the product of crosses between tetraploid and diploid plants (Terada and Masuda, 1943; Kihara and Nishiyama, 1947; Kihara, 1951). Diploid seedless watermelons have also been produced by pollination with soft X-ray irradiated pollen (Sugiyama and Morishita, 2000; Sugiyama *et al*., 2002 a). Using irradiated pollen is advantageous because seedless watermelons can be produced with ordinary cultivation methods due to the use of diploid plants. However, mass production of seedless watermelon seed by this method will require the production, irradiation, preservation and storage of a lot of pollen. Pollen viability after freezing and low temperature storage at low relative humidity has been reported

in many plant species (Holman and Brubaker, 1926; McGuire, 1952; King, 1961; Fatmer and Barnett, 1974; Nath and Anderson, 1975; Anderson *et al.*, 1978). Miyaji and Shirazawa (1977) reported that the best storage conditions for watermelon pollen are 5°C at 20 to 40% humidity, which extended the viability of pollen to 60 days on germination medium, but did not investigate fruit set. Watermelon pollen had fruit setting ability for 13 days when stored at 5°C under dry conditions (silica gel) (Araki *et al*., 1987). Pollen stored under temperature and humidity control, tend to have rapid germination rate decreases (Miyaji and Shirazawa, 1977; Araki *et al*., 1987; Sugiyama *et al*., 1998). Watermelon pollen storage in organic solvents has also been investigated (Kodani and Omura, 1981; Shimizu, 1983; Sugiyama *et al.*, 2002 b). Among the organic solvents, ethyl acetate and ethyl ether were the most suitable for watermelon pollen storage (Shimizu, 1983). Pollen stored in ethyl acetate at -20°C had a germination rate of over 40% for one month on germination medium (Morishita *et al.*, 2000), and fruit set was observed up to 79 days of storage in ethyl acetate at -20°C (Sugiyama *et al.*, 2002 b). These methods are effective for watermelon pollen, but are not suitable for long-term storage because of a decrease in vigor. Also, it is very difficult for farmers to recover and use pollen that has been stored in organic solvents. $N_2$, $CO_2$ and/or $O_2$ gas have been used as a general storage medium in the food industry, but there are few reports using gas for pollen storage. In this study, we describe effective new pollen storage method using atmospheric gases.

## 2. Materials and Methods

*Storage conditions for watermelon pollen*

Pollen from watermelon cultivar Green seeded was collected in October 2005 by cutting the anthers from the flowers and shaking the contents through a stainless steel filter into a stainless steel cup. Collected pollen was packed in paraffin paper and irradiated with 600 Gy soft X-ray (Soft X-ray Unit OM-60R, OHMIC Ltd.) at 14.5 Gy·min$^{-1}$. Irradiated pollen aliquots of about 1 g each were packed with a vacuum packaging machine ('TOSPACK' V-380G, TOSEI, Shizuoka, Japan), in air or under vacuum storage at 25°C, 4°C or -25°C for storage over 35 days. A second similar storage test was initiated in May 2007 to investigate effect of atmospheric gases on storage. Irradiated pollen aliquots of about 3 g each were packed with a vacuum packaging machine under $N_2$, $O_2$, $CO_2$, air or vacuum for storage at 25°C for 1-6 days.

Viability of the stored pollen was judged on the basis of germination on an artificial medium consisting of 14% sucrose, 0.1% boric acid, and 1.5% agar (WAKO, Ltd, Osaka, Japan). Germination percentages were determined after 3 hour incubation at 25°C by counting random samples of 100 pollen or more for each storage condition.

The first test was replicated 8 times, and the second test was replicated 5 times. Tukey's multiple-range test ($p<0.05$) on statistical software (Statcel, oms-publishing, Saitama, Japan) was used to test differences between treatment means.

*Fruit set ability of stored pollen, and fruits quality using stored pollen*

Experiments were performed at the Hokkaido Research Center, Sapporo, Japan. During cultivation, greenhouse temperatures were recorded ('Ondotori', T&D Corporation, Nagano, Japan), and monthly mean temperatures were determined. The average temperatures during cultivation in the greenhouse were 25°C in July, 30.4°C in August, and 25.1°C in September. Watermelon cultivar Fujihikari TR seeds were sown in pots on June 15, 2006 in a greenhouse. Seedlings with 5 to 6 leaves were transplanted 50 cm apart in a bed (2.3 x 35 m) in a greenhouse on July 10, 2006. The bed was covered with black and gray polyethylene mulch and fertilized with 9N-9P-9K (in kg·ha$^{-1}$) before transplanting. Plants were topped at the five leaf stage and three lateral vines were allowed to grow. Male flowers were pollinated with a paint brash at about the 15$^{th}$ node of lateral branches. All treatments were arranged in a randomized complete-block design with four single plant replications. Four fruits were harvested for each treatment. Values were compared by Tukey's multiple-range test ($p<0.05$).

*Source of pollen*

Watermelon cultivar Green Seeded seeds were sown as a source of pollen for the storage experiments in February 2006, before 'Fujihikari TR' was sown. Male flowers of 'Green seeded' were harvested in the morning to obtain pollen that would be stored for 14, 28 and 90 days, from April through July. Collected pollen was irradiated with 600 Gy soft X-ray and packed with a vacuum packaging machine either under $N_2$ gas or vacuum storage at 4 or -25°C for 14, 28 or 90 days. Soft X-ray irradiated pollen processed on the morning of the experiment was

used as a non-storage control. The stored pollen was used to pollinate female flowers of 'Fujihikari TR'. Female flowers of 'Fujihikari TR' were bagged with cellophane before anthesis. Prepared irradiated pollen was applied with a brush. After pollination, female flowers were covered again with cellophane bags to prevent contact with insect-borne pollen for about 3 days. Pollination occurred during the period of August 2-6. One or two female flowers on each of the 3 lateral shoots were pollinated from each storage treatment, and fruit set was confirmed about 7 days later. The fruit set rate for each plant was calculated. Fruits were selectively thinned, leaving one fruit per plant to mature for each treatment. Mature fruits were harvested after about 42 days. After harvesting, fruit weight, shape, rind thickness, flesh color, sugar content (Brix) and number of empty seeds exceeding 6 mm in length were recorded for each fruit. Fruit shape index is expressed as the ratio of length from peduncle to blossom end, to equatorial diameter. Mature harvested watermelons were cut in half, and flesh color was measured with a colorimeter [a*= Hue relates to red (+60) - green (-60) color axes, Nihondenshokukogyo, Tokyo, Japan].

The germination ability of stored pollen was assayed as in experiment 1 before pollination (the period of August 2-6). Fruit set and pollen germination rates were compared by Tukey's multiple-range test ($p<0.05$).

## 3. Results and Discussion

*Storage condition for watermelon pollen*

Pollen germination rates decreased rapidly at 25°C to nearly zero after 7 days (Fig. 1). When stored in atmospheric air at 4°C, the germination rate was significantly lower than with other treatments (except at 25°C) after 21 days. After 35 days of storage under vacuum at 4°C, pollen viability was significantly lower than either treatment at -25°C ($p<0.05$). Thus, high temperature is not suitable for storage of pollen, and packing pollen under vacuum at 4°C prolonged viability.

The viability of watermelon pollen stored under vacuum or $O_2$ at 25°C decreased during storage to levels that make $O_2$ and vacuum conditions unacceptable as atmospheric media for pollen storage (Table 1). Pollen stored for 7 days under $N_2$ and $CO_2$ showed good germination, indicating that storage of soft X-ray irradiated watermelon pollen under $N_2$ or $CO_2$ would enhance its viability.

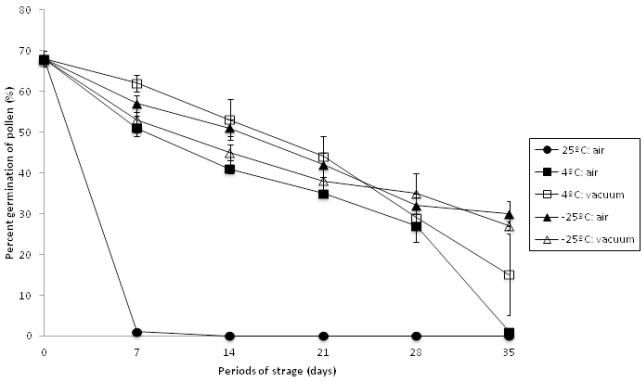



Fig. 1 - Germination of pollen after storage at different temperature and treatments. Bars indicate ± standard errors (n=8).

Table 1 - The differences of germination ability for cultured periods and treatments at 25°C

| Treatments | Periods (days) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| $N_2$ | 25.1 a [z] | 21.2 a | 16.5 a | 13.8 a | 7.9 a |
| $O_2$ | 11.2 b | 4.7 b | 0.0 d | 0.0 c | 0.0 b |
| $CO_2$ | 19.4 ab | 17.6 ab | 16.3 a | 15.1 a | 6.9 a |
| Vacuum | 16.9 ab | 10.9 b | 2.0 c | 1.7 b | 0.0 b |
| air | 20.3 ab | 19.5 ab | 9.3 b | 3.3 b | 0.2 b |

[z] Means followed by the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

*Fruit set and quality produced from stored pollen*

The ability of stored polled to germinate, and to produce agronomically acceptable fruit are two distinct qualities, both of which are necessary for the adoption of soft X-ray irradiated pollen on a commercial scale. There were observable differences in fruit set between pollen stored at 4°C and -25°C (Table 2). Lower temperatures resulted in good germination rates, and pollen which had been stored at -25°C had a longer shelf-life than at 4°C. Pollen stored at 4°C for

Table 2 - Relationship between storage condition and fruit set

| Treatment | Storage | | Pollination | Fruiting | Fruit set (%) |
|---|---|---|---|---|---|
| | Temperature (°C) | Period (days) | | | |
| Control | | 0 | 9 | 8 | 88.9 |
| Nitrogen | 4 | 14 | 17 | 17 | 100.0 |
| Nitrogen | 4 | 28 | 18 | 7 | 38.9 |
| Nitrogen | 4 | 90 | 16 | 0 | 0.0 |
| Nitrogen | -25 | 14 | 11 | 10 | 90.9 |
| Nitrogen | -25 | 28 | 16 | 14 | 87.5 |
| Nitrogen | -25 | 90 | 16 | 15 | 93.8 |
| Vacuum | 4 | 14 | 17 | 11 | 64.7 |
| Vacuum | 4 | 28 | 7 | 0 | 0.0 |
| Vacuum | 4 | 90 | 13 | 0 | 0.0 |
| Vacuum | -25 | 14 | 15 | 15 | 100.0 |
| Vacuum | -25 | 28 | 15 | 14 | 93.3 |
| Vacuum | -25 | 90 | 15 | 13 | 86.7 |

90 days did not set fruit whether or not it was stored under $N_2$ or vacuum. Although germination rates under $N_2$ and vacuum storage were almost the same, fruit set with pollen stored at 4°C for 14 or 28 days was more effective under $N_2$ than under vacuum. Pollen stored at 4°C for 28 days under $N_2$ had the ability to set fruit, but pollen stored at 4°C under vacuum did not. Snope and Ellison (1963) also reported that germination from freeze-dried pollen that had been stored under $N_2$ was got good. These results indicate that pollen vigor is maintained more effectively under $N_2$ than under vacuum. The percentage of germinating pollen stored at -25°C for 28 days was 24.8% and there was fruit set following hand pollination. However, the percentage of germinated pollen stored at 4°C for 28 days was 22.8%, but no fruit was set with this treatment. $N_2$ gas was an effective atmospheric medium for storage at 4°C in both tests. There were many reports that low temperature provides a good environment for stored pollen (Araki *et al*., 1987; Sugiyama *et al.*, 1998; Morishita *et al.*, 2000). Low temperature provides the major benefit, with storage in $N_2$ gas as an adjunct for pollen storage. Inert gases may be effective for the maintenance of pollen vitality because they may suppress metabolism of the pollen by displacing oxygen. The additive effect of $N_2$ with low temperature suggests that low metabolic rates are the key factor in maintaining pollen viability over time.

There was no significant difference in weight or shape between control fruit and fruit produced by pollen under any of the storage conditions (Table 3). Rind thickness and flesh color were also the same, except for fruits produced with pollen stored at -25°C for 90 or 14 days, respectively. Although the Brix of fruit from pollen stored at -25°C for 14 days was the lowest, there was no consistent relationship between storage conditions and Brix. Sugiyama and Morishita (2000) has observed that the number of empty seeds differs widely in individual fruits. In this experiment there were significant differences in the number of empty seeds, but there was no apparent relationship between empty seed numbers and storage conditions. Thus, there is no consistent relationship between pollen storage conditions and fruit quality. Further statistical analysis of the relationships between treatment, temperature and periods of pollen storage, was done for the survival of pollen, but there were no statistically significant differences.

Organic solvents are useful as a pollen storage method (Kodani and Omura, 1981). However, organic solvents carry the risk of environmental pollution and are difficult to use outside the laboratory. Storage of pollen at low temperatures and under $N_2$ eliminates the need for organic solvents, thus providing an eco-friendly storage solution which should be applicable for pollen export or import. Furthermore, pollen stored under $N_2$ at 25°C for 5 days had germination ability (Table 1) and then the temperature in the greenhouse was around 25-30°C, so it might be also no problem to bring the pollen from freezer to the greenhouse during pollination.

Table 3 - Fruit quality of seedless watermelon pollination with irradiated pollen in several conditions

| Treatment | Storage | | Fruit weight (kg) | Flesh shape [z] | Thickness of rind (mm) | Flesh color | Brix (%) | No. of empty seeds |
|---|---|---|---|---|---|---|---|---|
| | Temperature (°C) | Period (days) | | | | | | |
| Control | | 0 | 5.5±1.1[y] | 1.14 | 13.9±0.0 a [x] | 21.4±1.1 a | 10.3 a | 96.5±12.1 b |
| Nitrogen | 4 | 14 | 4.6±0.6 | 1.11 | 13.2±1.0 a | 20.4±1.7 a | 9.9 a | 102.5±86.3 b |
| Nitrogen | 4 | 28 | 4.9±0.5 | 1.12 | 13.5±0.2 a | 23.9±2.8 a | 9.7 b | 104.5±17.6 b |
| Nitrogen | -25 | 14 | 5.1±1.0 | 1.11 | 12.2±1.0 a | 18.4±3.6 b | 9.6 bc | 150.0±88.3 a |
| Nitrogen | -25 | 28 | 5.5±0.9 | 1.10 | 13.5±0.6 a | 23.9±1.5 a | 9.9 a | 104.8±25.2 b |
| Nitrogen | -25 | 90 | 5.2±0.5 | 1.14 | 11.9±0.4 b | 22.5±1.0 a | 9.7 b | 222.8±71.7 a |
| Vacuum | 4 | 14 | 4.9±0.3 | 1.13 | 12.2±1.4 a | 24.0±3.9 a | 9.7 b | 103.0±44.9 b |
| Vacuum | -25 | 14 | 5.4±0.5 | 1.10 | 12.7±1.9 a | 20.6±2.5 a | 10.2 a | 134.8±21.1a |
| Vacuum | -25 | 28 | 5.1±1.5 | 1.15 | 12.3±1.6 a | 21.0±1.9 a | 10.1 a | 158.5±81.0a |
| Vacuum | -25 | 90 | 5.7±0.4 | 1.17 | 12.5±0.6 a | 22.5±0.6 a | 10.0 a | 127.0±24.5b |

[z] Flesh shape is expressed as the ratio of height to width.

[y] Mean ± SE.

[x] Value within columns with the same letter are not significantly different (5% level).

Means followed by the same letter are not significantly different at the 5% level by Tukey's multiple-range test.

Plant breeders may also be able to overcome differences in crop or varietal flowering times by storing pollen with this method.

## References


ANDERSON J.O., NATH J., HARNER E.J., 1978 - *Effects of freeze-preservation on some enzyme. II. Freezing and freeze-drying stresses*. - Cryobiology, 15: 469-477.

ARAKI M., HASHIZUME T., HAGIHARA T., 1987 - *Effects of stored watermelon pollen on fruit set*. - J. Jpn. Soc. Hort. Sci., 56(2): 344-345.

FATMER R.E. Jr., BARNETT P.E., 1974 - *Low-temperature storage of black walnut pollen*. - Cryobiolgy, 11: 366-367.

HOLMAN R.M., BRUBAKER F., 1926 - *On the longevity of pollen*. - Univ. Cal. Publ. Bot., 13: 179-204.

KIHARA H., 1951 - *Triploid watermelon*. - Proc. Amer. Soc. Hort. Sci., 58: 217-230.

KIHARA H., NISHIYAMA I., 1947 - *An application of sterility of autotriploids to the breeding of seedless watermelons*. - Seiken Ziho, 3: 93-103. In Japanese with English summary.

KING J.R., 1961 - *The freez-drying of pollens*. - Econ. Bot., 15(1): 91-98.

KODANI T., OMURA S., 1981 - *On the collection and storage of pollens of watermelon by the utilization organic solvents*. - Bulletin of Tottori Agri. Exp. Stn., 2: 35-46. In Japanese with English summary.

McGUIRE DC., 1952 - *Storage of tomato pollen*. - Proc. Amer. Soc. Hort. Sci., 60: 419-424.

MIYAJI R., SHIRAZAWA M., 1977 - *Influence of temperature and the humidity for melon's and watermelon's pollens on their storage*. - Bulletin of the Kagoshima Agric. Exp. Stn., 5: 203-206.

MORISHITA M., HORI T., SAKATA Y., SUGIYAMA M., 2000 - *Storage of pollen irradiated with soft X-ray for producing seedless watermelon*. - J. Jpn. Soc. Hort. Sci., 69(2): 346.

NATH J., ANDERSON J.O., 1975 - *Effect of freezing and freeze-drying on the viability and storage of* Lilium longiflorum *L. and* Zea mays *L. Pollen*. - Cryobiology, 12: 81-88.

SHIMIZU T., 1983 - *Studies on the artificial pollination of watermelon. 1. 0n the storage of pollens of watermelon by the utilization of organic solvents*. - Bulletin of the Tottori Agric. Exp. Stn., 4: 9-17.

SNOPE J.A., ELLISON J.H., 1963 - *Storage of asparagus pollen under various conditions of temperature, humidity and pressure.* - Amer. Soc. Hort. Sci., 83: 447-452.

SUGIYAMA K., MORISHITA M., 2000 - *Production of seedless watermelon using soft-X-irradiated pollen*. - Scientia Horticulturae, 84: 255-264.

SUGIYAMA K., MORISHITA M., NISHINO E., 2002 a - *Seedless watermelons produced via soft-X-irradiated pollen*. - HortScience, 37: 292-295.

SUGIYAMA K., MORISHITA M., SAKATA Y., 1998 - *Storage of soft-X-irradiated pollen and effects of stored pollen on fruit quality of seedless watermelon*. - J. Jpn. Soc. Hort. Sci., 67(2): 283.

SUGIYAMA M., SAKATA Y., KITADANI E., MORISHITA M., SUGIYAMA K., 2002 b - *Pollen storage for production of seedless watermelon (*Citrullus lanatus*) using soft-X-irradiated pollen*. - Acta Horticulturae, 588: 269-272.

TERADA J., MASUDA K., 1943 - *Parthenocarpy of triploid watermelon*. - Agric. Hort., 18: 15-16.

AHS
Advances in Horticultural Science

# The impact of *Sinorhizobium meliloti* and *Pseudomonas fluorescens* on growth, seed yield and biochemical product of fenugreek under water deficit stress

**S. Bolandnazar [1 (*)], A. Sharghi [2], H. Naghdi Badhi [3], A. Mehrafarin [3], M.R. Sarikhani [4]**

[1] *Department of Horticulture, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.*
[2] *Department of Horticulture Science, Islamic Azad University, Science and Research Branch, Teheran, Iran.*
[3] *Medicinal Plants Research Centre, Institute of Medicinal Plants, ACECR, Karaj, Iran.*
[4] *Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.*

OPEN ACCESS

**(*) Corresponding author:**
bolandnazar@tabrizu.ac.ir

**Citation:**
BOLANDNAZAR S., SHARGHI A., NAGHDI BADHI H., MEHRAFARIN A., SARIKHANI M.R., 2018 - *The impact of Sinorhizobium meliloti and Pseudomonas fluorescens on growth, seed yield and biochemical product of fenugreek under water deficit stress.* - Adv. Hort. Sci., 32(1): 19-26



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 12 September 2017
Accepted for publication 8 November 2017

*Key words:* nicotinic acid, PGPR, seed, trigonelline, water use efficiency.

**Abstract: Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). For a long-serving period, the PGPRs have been applied as biofertilizers in crops culture. Recent studies indicated the importance of PGPR for controlling the water deficit. The present study investigates the effects of two different PGPRs on some morpho-physiological characteristics in fenugreek under water deficit stress. The first factor was application of four PGPR levels including (*1. Sinorhizobium meliloti, 2. Pseudomonas fluorescens*, 3. combination of *S. meliloti* and *P. fluorescens* and 4. control without bacterial inoculation) and four levels of soil water content including 40%, 60%, 80% and 100% of field capacity (FC) was considered as second factor. The results showed that, leaf area, shoot fresh and dry weight, nitrogen, phosphorus and potassium content, and water use efficacy (WUE) were significantly improved by PGPR inoculation and individual use of PGPR was more effective. Decreasing of soil water content up to 0.40 FC and inoculation of two bacteria led to increase of secondary metabolites such as nicotinic acid and trigonelline. However seed yield was decreased in PGPR treated plants.**

## 1. Introduction

Insufficient water induces a stress in plants called water deficit stress (Dodd and Ryan, 2016). Water deficit stress has major effects on plant growth and development, limiting crop production in the worldwide.

Water deficit negatively affects the plant growth and reproduction and disrupts the whole-plant functions (Bray, 2004; Hummel *et al*., 2010). It causes cellular changes such as solutes concentration, cell volume alteration, disruption of water potential gradients, changes in membrane shape and disrupting its integrity, loss in turgor pressure, and protein denaturation (Bray, 1997; Bartels and Sunkar, 2005). Water deficit is a major threat to agricultural production and tolerance to drought conditions is one of the main targets for crop improvement (Salekdeh *et al*., 2009).

Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria which constitute symbiotic relationships in large varieties of plants and are used as a biofertilizer (Shaukat *et al*., 2006). PGPR have been reported to confer positive effects and induce plant resistances to environmental stresses and diseases caused by pathogens (Kloepper *et al*., 2004; Mayak *et al*., 2004 a, b; Compant *et al*., 2005; He and Yang, 2007; Dimkpa *et al*., 2009; Yang *et al*., 2009). A wide variety of mechanisms that can improve the plant growth, have been suggested to be impressed by PGPR. These involved mechanisms are as follows: nitrogen fixation (Van Loon, 2007), production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC) (Govindasamy *et al*., 2008), production of volatile organic compounds (Ryu *et al*., 2003), induction of systemic resistance (Chandler *et al*., 2008), phytohormone production (Vessey, 2003), siderophore production (El-Tarabily and Sivasithamparam, 2006), phosphate solubilization (Ryu *et al*., 2003) and potassium releasing (Sarikhani *et al*., 2016).

Fenugreek (*Trigonella foenum-graecum* L.) is a member of the Fabaceae family, cultivated worldwide as a semiarid crop, and traditionally used as a medicinal plant. Fenugreek is grown as a spice and a vegetable crop and also have been used as a traditional therapy for the remedy of diabetes (Miraldi *et al*., 2001; Smith, 2003; Fernández-Aparicio *et al*., 2008). Its effect as an antidiabetic and antiatherosclerotic have been documented (Ajabnoor and Tilmisany, 1988; Sharma and Raghuram, 1990). Fenugreek's leaves are a rich source of iron, calcium, β-carotene and other vitamins and its seeds contain tannic acid, diosgenin, trigocoumarin, alkaloids trigonelline, trigomethyl coumarin, gitogenin and vitamin A (Warke *et al*., 2011). Recently, published literatures indicated that PGPR ameliorate the plants tolerance to abiotic stresses through a variety of mechanisms (Srivastava *et al*., 2008; Sandhya *et al*., 2010). Also beneficial effects of PGPRs on medicinal plants have been reported (Jaleel *et al*., 2007).

Shafighi *et al*. (2014) reported that inoculation of fenugreek with PGPR increases plant height, vegetative growth and seed yield in both well watered and water limited condition. Rubin *et al.* (2017) emphasized that application of PGPR decreases abiotic stress especially drought stress in various plants. They summarized that PGPR inoculation not only improves root and shoot biomass in non-stress condition but also it can enhance aerial biomass and reproductive yield under drought stress condition, however root mass was not increased under stress. In enormous studies, the synergistic effect of nitrogen fixing bacteria especially Rhizobia and phosphate solubilizing bacteria such as *Pseudomons* has been reported but application of these PGPR in stressed condition needs more attention. Therefore, in this study we focused to the inoculation effect of two native and endogenous PGPR (*Sinorhizobium meliloti* Tabriz and *Pseudomonas fluorescens* Tabriz) in fenugreek under drought stress condition.

## 2. Materials and Methods

The seeds of fenugreek with good germination quality was provided from "Jahad Daneshgahi-Iranian Institute of Medicinal Plants", Karaj, Iran. The present investigation, carried out in research greenhouse of Faculty of Agriculture at the University of Tabriz during 2015-2016. Two bacteria, including *Pseudomonas fluorescens* Tabriz and *Sinorhizobium meliloti* Tabriz were obtained from the Laboratory of Soil Biology, University of Tabriz (Tabriz, Iran). Nutrient Broth (NB) and Yeast Mannitol Broth (YMB) were used to prepare a primary culture of *Pseudomonas* and *Sinorhizobium* respectively to inoculate seeds of plant. The experiments were conducted in a factorial design based on completely randomized block design with three replications. The first factor was application of PGPR in 4 levels including 1. *S. meliloti,* as nitrogen fixing bacterium 2. *P. fluorescens,* as phosphorous solubilizing bacterium 3. combination of *S. meliloti* and *P. fluorescens* 4. negative control without any bacteria and fertilizer treatment. The second factor was soil water content treatment based on field capacity (FC) in 4 levels (100, 80, 60 and 40% of FC). Seed of fenugreek was sown in a plastic pot which had 5 kg soil and after establishment 5 plants remained in each pot. Soil water content was maintained as aforementioned values by daily weighting of pots by digital scale and water loss by evapotran-

spiration was added to each pot. Plants kept in a greenhouse under a 16 h photoperiod, 24±4/18±3°C day/night temperatures, and 40-60% relative humidity. At the end of the experiment leaf area was measured, by the leaf area meter (LI 3100C area meter, LI-COR, USA). Dry weight of each part was determined after drying at 72˚C until constant weight. The fresh and dry weight plants were determined using a digital weighing scale.

The composition of potassium and phosphorus was determined by nitric perchloric and nitric acid digestion methods (Zasoski and Burau, 1977; Havlin and Soltanpour, 1980). Phosphorous was measured by a vanadate-molybdate method using a spectrophotometer (Motic, CL-45240-00, China) and K was determined using a flame photometer (Model 405G, Iran). Nitrogen was measured according to the Kjeldahl method that involves changing the form of organic nitrogen to ammonium ($NH_4$) by concentrated sulfuric acid and then measuring the amount of ammonium production (Baker and Thompson, 1997). Also the seed yield was recorded at maturity. Water use efficiency (WUE) was calculated by the following formula:

$$WUE = DW/UW$$

In this formula, DW and UW represent dry mass production and the amount of consumed water, respectively (Karimi and Roosta, 2014).

*Analysis and quantization of trigonelline*

Trigonelline in the seed sample was measured according to modified method of Zheng and Ashihara (2004). The samples were ground with 80% methanol and magnesium oxide (MgO) in a mortar and pestle. After incubation at 60°C for 30 min, the homogenates were centrifuged and the supernatant was collected. After complete evaporation of methanol, the methanol-soluble extracts were dissolved in distilled water. The samples were filtered using a disposable syringe filter unit and the aliquots were used for determination of trigonelline (TG) by HPLC. The analyses of the samples were carried out using a Knauer K2600A liquid chromatography (Germany), equipped with a Nucleosil C18 (150 mm × 4.6 mm I.D, 5 µm) column. A mixture of methanol: water (50:50 v/v) served as the mobile phase and pH of solution adjusted to 5.0 with 50 mM sodium acetate. The elution has been made in an isocratic mode at a flow rate of 1 mL $min^{-1}$ and the detection made at 268 nm by UV detector from the above mentioned company (Koshiro *et al*., 2006). One analysis requires 20 min. The retention time of this alkaloid was 4.4 min. Before carrying out HPLC analysis, we made calibration curve by using different concentrations (0.1, 0.2, 0.5, 0.7 and 1.0 mg $mL^{-1}$) of trigonelline in phase media. Then calibration curve made with trigonelline and the correlations were excellent for trigonelline. This process was performed according to United States Pharmacopoeia (U.S. Pharmacopeia, USA) by cold extraction method as directed for alcohol soluble material, except where water was used in place of alcohol.

*Measuring nicotinic acid*

For the measurement of nicotinic acid, it was carried out according to modified Martin *et al*. (1997). The 0.5 g of fenugreek seed powder was mixed with 0.5 g of magnesium oxide (MgO) and 30 ml of distilled water was added to it. The resulting mixture for 30 minutes at 100°C was placed in bath water bath. After cooling, the resulting mixture was filtered using filter paper (1) and was brought to a volume of 50 ml with distilled water. Finally absorption at a wavelength of 263 nm of the samples was measured by a spectrophotometer. Nicotinic acid concentrations were determined using the standard curve.

*Statistical analysis*

All collected data were subjected to two-way analysis of variance (ANOVA) through PROC GLM procedure, using a SAS statistical package (SAS Institute, software Version 9.4, Cary, NC, USA). If interactions were significant, means were compared by Duncan's multiple range test to determine whether means of the dependent variable were significantly different at $P<0.05$.

## 3. Results

Analysis of data variances indicated that the effect of PGPR and soil water content and their interaction on leaf area, shoot fresh and dry weight were significant ($P\leq0.01$). Means comparison showed that PGPR inoculation increased fenugreek leaf area, shoot dry and fresh weight especially *S. meliloti* (Table 1). By increasing of water deficit stress, leaf area, shoot fresh and dry weight was decreased (Table 2). In aspect of interaction between PGPR inoculation and water stress, the highest and lowest leaf area, shoot fresh and dry weight was observed in well watered (100% FC) and combination of *S. meliloti* and *P. fluorescens* treated plants and severe water stressed control plants respectively (Fig. 1, 2 and 3). It seems that in normal condition *S. meliloti*

Table 1 - Results of mean comparison of different PGPR treatments

| Bacteria | Leaf area ($cm^2$) | Shoot fresh weight (g) | Shoot dry weight (g) | N ($mg\ g^{-1}$) | P ($mg\ g^{-1}$) | K ($mg\ g^{-1}$) | WUE ($g\ kg^{-1}$) | Seed yield ($g\ pot^{-1}$) | Trigonelline ($mg\ g^{-1}$) | Nicotinic acid ($mg\ g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 756 c | 17.71 c | 4.18 c | 7.36 c | 0.54 d | 25.84 c | 0.139 c | 26.53 a | 6.84 b | 12.34 ab |
| *S. meliloti* (S) | 1212 a | 32.14 a | 6.00 a | 14.27 a | 0.71 a | 31.73 a | 0.226 a | 14.23 b | 6.97 b | 12.69 b |
| *P. fluorescens* (P) | 959 ab | 29.74 a | 4.37 b | 12.93 b | 0.67 b | 30.40 b | 0.213 a | 13.25 c | 7.65 a | 14.11 a |
| SxP | 937 b | 21.14 b | 5.05 ab | 12.17 b | 0.63 c | 29.43 c | 0.181 b | 14.19 b | 7.74 a | 14.05 a |

SxP= treatment containing both *S. meliloti* and *P. fluorescens*.
Dissimilar letters indicating significant differences (Duncan's multiple range test P≤0.01). N, P and K were measured in dry shoot of plant.

Table 2 - Results of mean comparison of different irrigation treatments

| Soil water content (FC) | Leaf area ($cm^2$) | Shoot fresh weight (g) | Shoot dry weight (g) | N ($mg\ g^{-1}$) | P ($mg\ g^{-1}$) | K ($mg\ g^{-1}$) | WUE ($g\ kg^{-1}$) | Seed yield ($g\ pot^{-1}$) | Trigonelline ($mg\ g^{-1}$) | Nicotinic acid ($mg\ g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 100% | 1189 a | 33.78 a | 6.48 a | 13.60 a | 0.63 c | 29.35 b | 0.138 c | 24.18 a | 6.26 c | 11.10 d |
| 80% | 1025 b | 24.76 b | 5.79 b | 12.20 b | 0.59 d | 29.05 b | 0.165 b | 23.52 b | 6.63 c | 12.07 c |
| 60% | 913 c | 18.17 c | 3.97 c | 12.19 b | 0.65 b | 30.25 a | 0.169 b | 12.83 c | 7.55 b | 13.84 b |
| 40% | 737 d | 13.93 d | 3.36 d | 9.73 c | 0.69 a | 30.37 a | 0.287 a | 10.20 d | 8.76 a | 16.18 a |

Dissimilar letters indicating significant differences (Duncan's multiple range test P≤0.01). N, P and K were measured in dry shoot of plant.

improved aerial growth of fenugreek better than *P. fluorescens*, whereas combination use of two PGPR bacteria was successful in enhancement of shoot growth better than individual using (Fig. 1).

Un-inoculated control plants produced significantly higher seed yield per pot than PGPR treated fenugreek (Table 1). Water limitation led to decrease in seed yield (Table 2). The maximum seed weight was observed in control plants (Fig. 4).

The nitrogen, phosphorus and potassium concentration was significantly affected by PGPRs and water deficit. As shown in the table of mean comparison (Table 1) for the effects of PGPR treatments, the highest N, P and K were observed in plants treated with *S. meliloti* follows by *P. fluorescens* and treat-

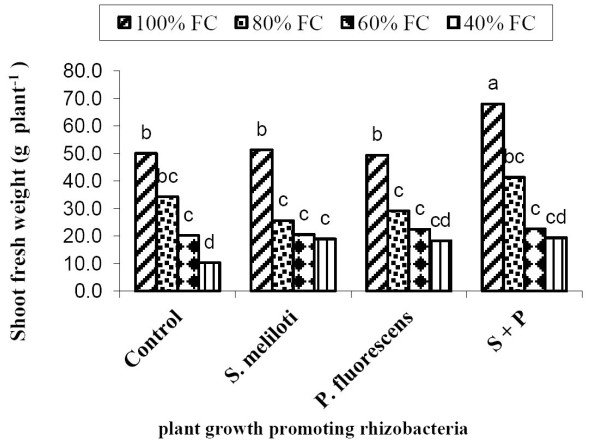



Fig. 2 - Interaction of PGPR and soil water content on the shoot fresh weight fenugreek.

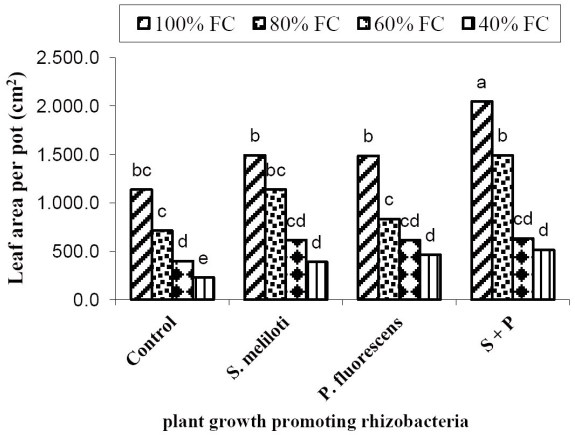



Fig. 1 - Interaction of PGPR and soil water content on leaf area of fenugreek.

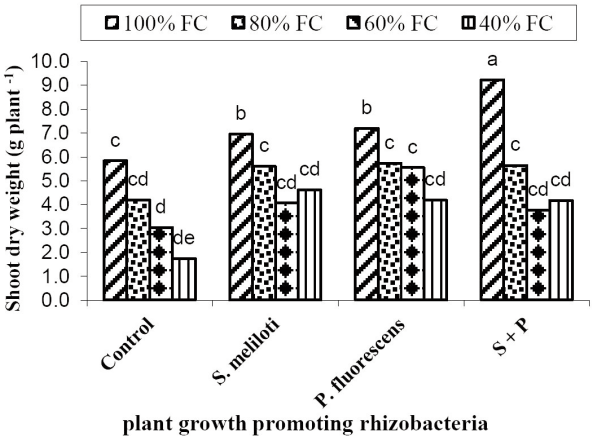



Fig. 3 - Interaction of PGPR and soil water content on the shoot dry weight of fenugreek.

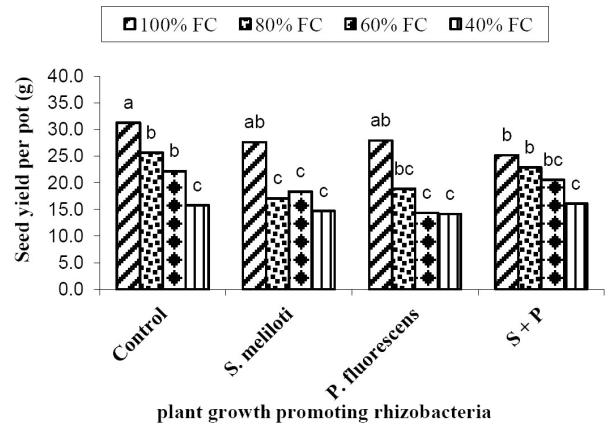



Fig. 4 - Interaction of PGPR and soil water content on the seed yield of fenugreek.

ments containing both *S. meliloti* and *P. fluorescens*. By decreasing of soil water content, N concentration was decreased significantly, but inverse, P and K concentration was increased significantly with an exception at 80% of FC treatment (Table 2). The highest and the lowest N concentration was observed in plants treated with *S. meliloti* and dual application of PGPR bacteria at well watered (100% FC) and control plant at severe water stress treatment (40% FC) respectively (Fig. 5) and the highest and the lowest P concentration was related to plants treated with *S. meliloti* and *P. fluorescens* in single form at well watered (100% FC) and control plant at severe water stress treatment (40% FC) respectively (Fig. 6).

According to the interaction effects between PGPRs and drought stress the highest and lowest K concentration was observed in dual application of PGPR bacteria at well watered (100% FC) and control plant under sever water stress (40% FC) respectively (Fig. 7).

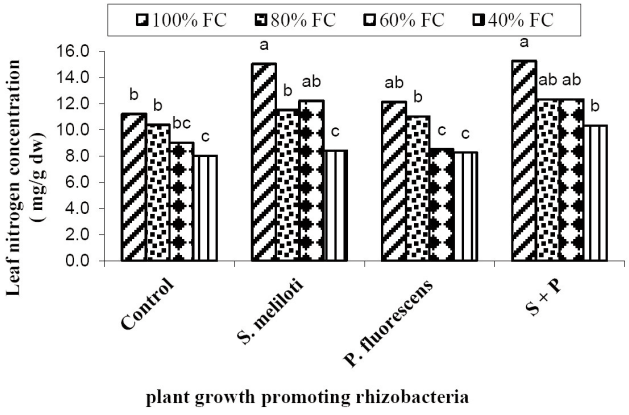



Fig. 5 - Interaction of PGPR and soil water content on N concentration of fenugreek shoot.

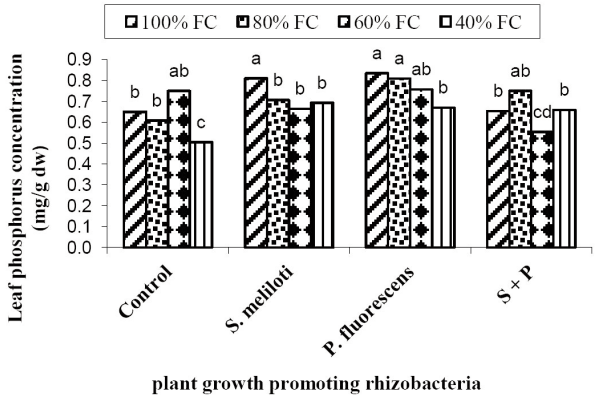



Fig. 6 - Interaction of PGPR and soil water content on P concentration of fenugreek shoot.

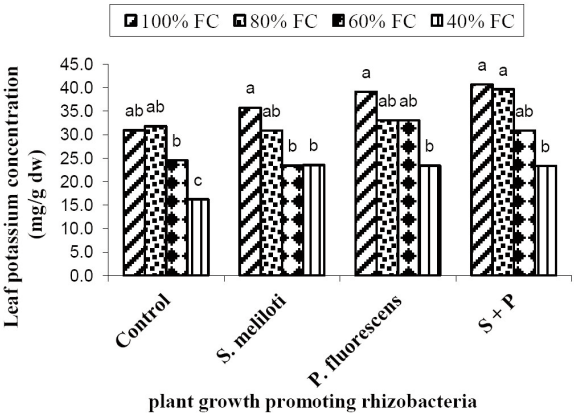



Fig. 7 - Interaction of PGPR and soil water content on K concentration of fenugreek shoot.

Water use efficiency (WUE) was significantly affected by PGPR and soil water content. Mean comparison indicated that PGPR inoculate plants produced more shoot biomass per water unit than control ones (Table 1). By increasing water deficit stress WUE was increased significantly (Table 2). In aspect of interaction between PGPR and water stress it was shown that both dual application of *P. fluorescens* and *S. meliloti* and separate application of bacteria under severe water deficit stress (40% FC) led to highest WUE and well watered control plants produced the lowest WUE (Fig. 8).

As it was shown in the table of mean comparison (Table 1), individual and dual PGPR treatments improved trigonelline and nicotinic acid. By increasing water deficit stress trigonelline and nicotinic acid was significantly increased (Table 2). The highest and lowest trigonelline and nicotinic acid was observed in *P. fluorescens* inoculated at 40% FC soil water content treatment and control in 100% FC soil water content treatment respectively (Figs. 9 and 10).

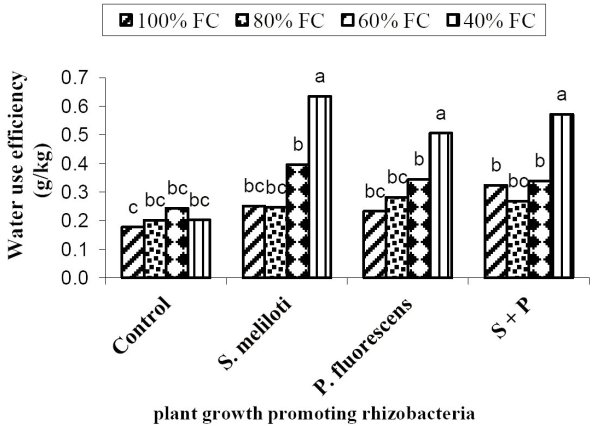



Fig. 8 - Interaction of PGPR and soil water content on the WUE of fenugreek.

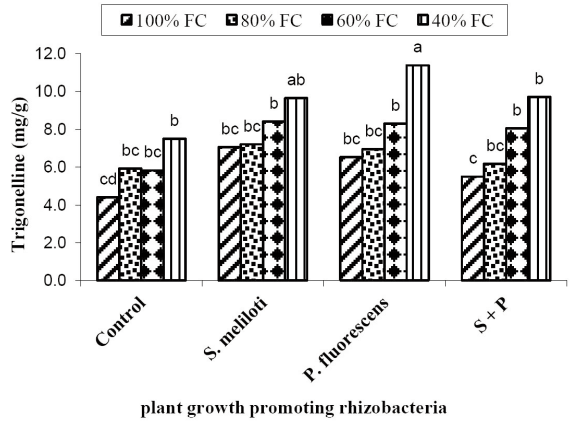



Fig. 9 - Interaction of PGPR and soil water content on the Trigonelline of fenugreek seed.

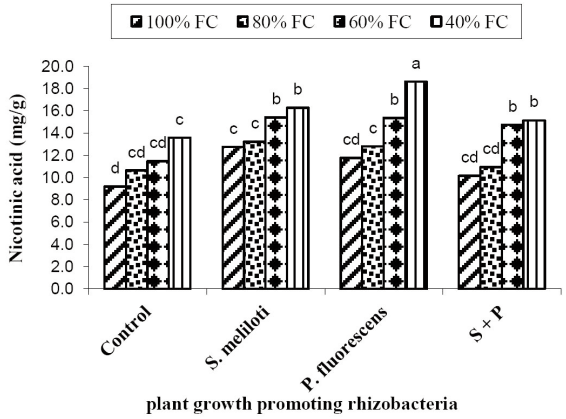



Fig. 10 - Interaction of PGPR and soil water content on nicotinic acid of fenugreek seed.

## 4. Discussion and Conclusions

Water deficit limits many crop production worldwide and negatively affects the plant growth and reproduction; recently published literatures indicated that PGPR ameliorate the plants tolerance to abiotic stresses through a variety of mechanisms (Srivastava *et al*., 2008; Sandhya *et al*., 2010). In keeping with our results, Mishra *et al*. (2010) indicated that PGPRs could ameliorate the negative effects of salinity stress conditions by positive effects on parameters such as increasing the germination in plants, and also increasing the yield, drought tolerance, and growth. It has also been reported that even in the presence of optimum levels of nitrogenous fertilizers, inoculating with PGPR containing ACC-deaminase activity can improve the yield and growth of inoculated plants (Shaharoona *et al*., 2006). According to the results of this investigation inoculation with PGPR containing ACC-deaminase considerably decreased the damages caused by drought stress on the growth and yield. They reported that un-inoculated plants exposed to drought stress at vegetative growth stage had significantly decreased shoot growth by 41%, while in the inoculated plants the decreased shoot growth was only by 18 per cent.

In present study control plants without any inoculation produced higher seed yield per pot than PGPR treated fenugreek (Table 1). It should be noted that the experiment duration was 5 month and fenugreek has indeterminate flowering habit, so while plants flowering were continued it was harvested. It have been reported that PGPR could delay the flowering time (Jaleel *et al*., 2007). Water stress led to decrease in fenugreek seed yield. The reason may be due to this fact that in the absence of stress conditions, more photosynthesis material have been stored in the organs such as stems and leaves which by transferring to the seeds increased the grain weight. In contrary, under stress conditions, the water and mineral absorption by the plant is disrupted which decreases the plant growth and reduces the transmission of photosynthesis material in leaf and other organs to the grain (Jaleel *et al*., 2007).

Similar to our findings, root bacterial inoculations significantly affected the plant nutrient element contents in apple compared to controls and increased the phosphorus content of treated plants (Karlidag *et al*., 2007). Ordookhani *et al*. (2010) reported that *P. fluorescens* improved potassium in tomato plant.

Water use efficiency was increased by PGPR in fenugreek in present investigation. Similar to the present findings Jaleel *et al*. (2007) demonstrated that the minimum WUE was related to un-inoculated cases which was improved by inoculation with *Rhizobium* and PGPR. In the present investigation leaf area, shoot weight, dry weight, nitrogen, phosphorus and potassium content, and WUE increased significantly by treatment with both PGPRs. PGPR

colonizes the plant's root system and modulates its growth through increasing the availability of nutrients it also protects the plants from phytopathogens (Lee *et al*., 2013). It has been reported that in induced drought stress condition, fixed nitrogen during photosynthesis, spend the production of secondary metabolites (Aliabadi Farahani *et al*., 2009). Also beneficial effects of PGPRs on medicinal plants have been reported (Jaleel *et al*., 2007). It has been reported that the synthesis of secondary metabolites in medicinal plants is induced specific pathway by the impact of microorganisms (Bouchereau *et al*., 1996). Integrative use of PGPRs and water deficit stress could be an enhance the eco-friendly strategy of PGPRs and plants and could increasing the alkaloid yields in medicinal plants (Lee *et al*., 2013). Since the fenugreek is used as a medicinal plant this strategy could be applied for increasing its useful secondary metabolites.

In conclusion, the results of the present investigation indicate that both *S. meliloti* and *P. fluorescens* could effectively increase vegetative growth, nitrogen, phosphorus and potassium content, secondary metabolites and WUE in fenugreek regardless of water stress. Also under water stress condition PGPR increased plant growth. However in present study seed yield because of delaying bolting time was decrease by application of PGPR.

**References**


AJABNOOR M.A., TILMISANY A.K., 1988 - *Effect of* Trigonella foenum graceum *on blood glucose levels in normal and alloxan-diabetic mice*. - J. Ethnopharmacol., 22: 45-49.

ALIABADI-FARAHANI H., VALADABADI S.A., DANESHIAN J., KHALVATI M.A., 2009 - *Evaluation changing of essential oil of balm (*Melissa officinalis *L.) under water deficit stress conditions.* - J. Med. Plants Res., 3: 329-333.

BAKER W.H., THOMPSON T.L., 1997 - *Determination of total nitrogen in plant samples by Kjeldahl.* - In: PLANK C.O. (ed.) *Plant analysis reference procedures for the southern region of the United States*. University of Georgia, Athens, USA, pp. 13-16.

BARTELS D., SUNKAR R., 2005 - *Drought and salt tolerance in plants.* - Crit. Rev. Plant Sci*.,* 24: 23-58.

BRAY E.A., 1997 - *Plant responses to water deficit.* - Trends Plant Sci*.,* 2: 48-54.

BRAY E.A., 2004 - *Genes commonly regulated by water-deficit stress in* Arabidopsis thaliana. - J. Exper. Bot*.,* 55: 2331-2341.

BOUCHEREAU A., CLOSSAIS B.N., BENSAOUD A., BEPORT L. RENAR M., 1996 - *Water stress effects on rapeseed quality*. - Europ. J. Agron., 5: 19-30.

CHANDLER D., DAVIDSON G., GRANT W., GREAVES J., TATCHELL G., 2008 - *Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability*. - Trends Food Sci. Technol., 19: 275-283.

COMPANT S., DUFFY B., NOWAK J., CLÉMENT C., BARKA E.A., 2005 - *Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects*. - Appl. Environ. Microbiol., 71: 4951-4959.

DIMKPA C., WEINAND T., ASCH F., 2009 - *Plant-rhizobacteria interactions alleviate abiotic stress conditions.* - Plant Cell Environ., 32: 1682-1694.

DODD I.C., RYAN A.C., 2016 - *Whole plant physiological responses to water deficit stress*. - Wiley & Sons Ltd, Chichester, UK., pp. 1-9.

EL-TARABILY K.A., SIVASITHAMPARAM K., 2006 - *Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters.* - Soil Biol. Biochem., 38: 1505-1520.

FERNÁNDEZ-APARICIO M., EMERAN A.A., RUBIALES D., 2008 - *Control of* Orobanche crenata *in legumes intercropped with fenufreek* (Trigonella foenum-graceum). - Crop Protec., 27: 653-659.

GOVINDASAMY V., SENTHILKUMAR M., GAIKWAD K., ANNAPURNA K., 2008 - *Isolation and characterization of ACC deaminase gene from two plant growth-promoting rhizobacteria*. - Curr. Microbiol., 57: 312-317.

HAVLIN J.L., SOLTANPOUR P., 1980 - *A nitric acid plant tissue digest method for use with inductively coupled plasma spectrometry 1*. - Commun. Soil Sci. Plant Anal., 11: 969-980.

HE Z.L., YANG X.E., 2007 - *Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils.* - J. Zhejiang Univ. Sci. B, 8: 192-207.

HUMMEL I., PANTIN F., SULPICE R., PIQUES M., ROLLAND G., DAUZAT M., CHRISTOPHE A., PERVENT M., BOUTEILLÉ M., STITT M., 2010 - Arabidopsis *plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme and gene expression analysis*. - Plant Physiol*.,* 154: 357-372.

JALEEL C.A., MANIVANNAN P., SANKAR B., KISHOREKUMAR A., GOPI R., SOMASUNDARAM R., PANNEERSELVAM R., 2007 - Pseudomonas fluorescens *enhances biomass yield and ajmalicine production in* Catharanthus roseus *under water deficit stress.* - Colloids Surf. B: Biointerfaces*,* 60: 7-11.

KARIMI H.R., ROOSTA H., 2014 - *Evaluation of inter-specific hybrid of* P. atlantica *and* P. vera L. cv. 'Badami riz-e-Zarand'*as pistachio rootstock to salinity stress according to some growth indices and eco-physiology and bichemichal parameters.* - J. Stress Physiol. Biochem., 10(3): 5-17.

KARLIDAG H., ESITKEN A., TURAN M., SAHIN F., 2007 - *Effects of root inoculation of plant growth promoting rhi-*

*zobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apple*. - Sci. Hortic., 114: 16-20.

KLOEPPER J., REDDY M., RODRÍGUEZ-KABANA R., KENNEY D., KOKALIS-BURELLE N., MARTINEZ-OCHOA N., VAVRINA C., 2004 - *Application for rhizobacteria in transplant production and yield enhancement*. - Acta Horticulturae, 631: 179-188.

KOSHIRO Y., ZHENG X.Q., WANG M., NAGAI C., ASHIHARA H., 2006 - *Changes in content and biosynthetic activity of caffeine and trigonelline during growth and ripening of* Coffea arabica *and* Coffea canephora. - Plant Sci., 171(2): 242-250.

LEE K.J., OH B.T., SERALATHAN K.K., 2013 - *Advances in plant growth promoting* Rhizobacteria *for biological control of plant diseases,* pp. 1-13. - In: MAHESHWARI D.K. (ed.) *Bacteria in agrobiology: Disease management*. Springer-Verlag, Berlin, Heidelberg, Germany, pp. 495.

MARTIN M.J., PABLES F., BELLE M.A., GONZALES A.G., 1997 - *Determination of trigonelline in green and roasted coffee from single column ionic chromatography*. - Fresenius J. Anal. Chem., 357: 357-358.

MAYAK S., TIROSH T., GLICK B.R., 2004 a - *Plant growth-promoting bacteria confer resistance in tomato plants to salt stress.* - Plant Physiol. Biochem., 42: 565-572.

MAYAK S., TIROSH T., GLICK B.R., 2004 b - *Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers.* - Plant Sci., 166: 525-530.

MIRALDI E., FERRI S., MOSTAGHIMI V., 2001 - *Botanical drugs and preparations in the traditional medicine of West Azerbaijan (Iran).* - J. Ethnopharmacol., 75: 77-87.

MISHRA M., KUMAR U., MISHRA P.K., PRAKASH V., 2010 - *Efficiency of plant growth promoting rhizobacteria for the enhancement of* Cicer arietinum *L. growth and germination under salinity.* - Adv. Biol. Res., 4: 92-96.

ORDOOKHANI K., KHAVAZI K., MOEZZI A., REJALI F., 2010 - *Influence of PGPR and AMF on antioxidant activity, lycopene and potassium contents in tomato*. - Afric. J. Agric. Res., 5: 1108-1116.

RYU C.M., FARAG M.A., HU C.H., REDD M.S., WEI H.X., PARÉ P.W., KLOEPPER J.W., 2003 - *Bacterial volatiles promote growth in* Arabidopsis. - Proceed. National. Acad. Sci., 100: 4927-4932.

RUBIN R.L., VAN GRONIGEN K.J., HUNGATE B.A. 2017 - *Plant growth promoting rhizobacteria are more effective under drought: a metha-analysis.* - Plant Soil, 416(1-2): 309-323.

SALEKDEH G.H., REYNOLDS M., BENNETT J., BOYER J., 2009 - *Conceptual framework for drought phenotyping during molecular breeding.* - Trends Plant Sci., 14: 488-496.

SANDHYA V., ALI S.Z., GROVER M., REDDY G., VENKATESWARLU B., 2010 - *Effect of plant growth promoting* Pseudomonas spp. *on compatible solutes, antioxidant status and plant growth of maize under drought stress*. - Plant Growth Regul., 62: 21-30.

SARIKHANI M.R., KHOSHRU B., OUSTAN S., 2016 - *Efficiency of some bacterial strains on potassium release from micas and phosphate solubilization under* in vitro *conditions*. - Geomicrobiol. J., 33(9): 832-838.

SHAFIGHI A., PAZOKI A., ASLI D.E., 2014 - *Alleviation of water stress in fenugreek (*Trigonella foenum-graceum *L.) using different PGPR application methodes.* - Adv. Environ. Biol., 8(24): 275-280.

SHAHAROONA B.M., ARSHAD Z., ZAHIR A., KHALID A., 2006 - *Performance of* Pseudomonas *spp. containing ACC-deaminase for improving growth and yield of maize* (Zea mays *L.) in the presence of nitrogenous fertilizer.* - Soil Biol. Biochem., 38: 2971-2975.

SHARMA R., RAGHURAM T., 1990 - *Hypoglycaemic effect of fenugreek seeds in non-insulin dependent diabetic subjects*. - Nutri. Res., 10: 731-739.

SHAUKAT K., AFFRASAYAB S., HASNAIN S., 2006 - *Growth responses of* Triticum aestivum *to plant growth promoting rhizobacteria used as a biofertilizer.* - Res. J. Microbiol., 1: 330-338.

SMITH M., 2003 - *Therapeutic applications of fenugreek*. - Altern. Med. Rev., 8: 20-27.

SRIVASTAVA S., YADAV A., SEEM K., MISHRA S., CHAUDHARY V., NAUTIYAL C., 2008 - *Effect of high temperature on* Pseudomonas putida *NBRI0987 biofilm formation and expression of stress sigma factor RpoS.* - Curr. Microbiol., 56: 453-457.

VAN LOON L., 2007 - *Plant responses to plant growth-promoting rhizobacteria*. - Europ. J. Plant Patholol., 119: 243-254.

VESSEY J.K., 2003 - *Plant growth promoting rhizobacteria as biofertilizers*. - Plant Soil, 255: 571-586.

WARKE V.B., DESHMUKH T.A., PATIL V.R., 2011 - *Development and validation of RP-HPLC method for estimation of diosgenin in pharmaceutical dosage form*. - Asian J. Pharm. Res., 4: 126-128.

YANG J., KLOEPPER J.W., RYU C.M., 2009 - *Rhizosphere bacteria help plants tolerate abiotic stress.* - Trends Plant Sci., 14: 1-4.

ZASOSKI R., BURAU R., 1977 - *A rapid nitric-perchloric acid digestion method for multi-element tissue analysis.* - Commun. Soil Sci. Plant Anal., 8: 425-436.

ZHENG X.Q., ASHIHARA H., 2004 - *Distribution, biosynthesis and function of purine and pyridine alkaloids in* Coffea arabica *seedlings*. - Plant Sci., 166: 807-813.

Adv. Hort. Sci., 2018 32(1): 27-32 DOI: 10.13128/ahs-21157

AHS
Advances in Horticultural Science

# Phenology and pomology of almond's cultivars and genotypes using multivariate analysis

**A. Imani [1], M. Shamili [2 (*)]**

[1] *Temperate Fruit Research Center, Horticultural Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaji, Iran.*

[2] *Horticulture Department Agriculture Faculty, University of Hormozgan, Bandar Abbas, Iran.*

*Key words:* flower, kernel, multiple regression, nut, path analysis, yield.



**(*) Corresponding author:**
shamili@ut.ac.ir

**Citation:**
IMANI A., SHAMILI M., 2018 - *Phenology and pomology of almond's cultivars and genotypes using multivariate analysis.* - Adv. Hort. Sci., 32(1): 27-32



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 14 August 2017
Accepted for publication 16 November 2017

**Abstract: The present research aimed to study the flower and fruit properties of 60 almond's cultivars and genotypes. All fruit and kernel traits had high heritability (ranged from 20.73 to 92.13%). Double kernel and pistil length showed the most and few genotypic variation, respectively. The most yield belonged to Filippo Ceo, K8-24, Fragiulo and K9-24 (6.9, 6.84, 6.78 and 7.6 kg/tree respectively). Multivariate analysis was the applied technique to determine the relationship among important traits. Kernel weigh caused yield directly and kernel width indirectly. To estimate kernel weight, kernel variables had less R square (0.61) than nut variables (0.94).**

## 1. Introduction

The efficiency of plants breeding program depends on selection of desirable parents and progenies. Almond cultivars differ in the growth and branching pattern, as well as bearing habit (Duval and Grasselly, 1994).

Self-compatibility (Socias i Company and Felipe, 1988; Ortega and Dicenta, 2003), late blooming (Vargas and Romero, 2001), flower density, production rate (Dicenta *et al.*, 1993 a; Gradziel and Kester, 1998), fruit maturity period (Kester and Asay, 1975; Dicenta *et al.,* 1993 b) and kernel properties (Spiegel-Roy and Kochba, 1974, 1981; Kester *et al.,* 1991) are the main almond breeding objectives, to be informed by the relationship among these traits, facilitates the breeding process, and selection of the desirable almond genotypes (Dicenta *et al.,* 1993 a).

Multivariate analysis is the common technique to evaluate almond genotypes according to quantitative and qualitative characteristics (De Giorgio and Polignano, 2001; De Giorgio *et al.*, 2007). Lansari *et al.* (1994) used this method in order to evaluate morphological variation of almonds varieties. Their results indicated that nut and kernel traits, compare to

vegetative characteristics, has important role in genotype distinction. De Giorgio and Polignano (2001) mentioned fruit characteristics are the most effective variables to differentiate almond genotypes. De Giorgio *et al.* (2007) divided 88 almond varieties, into seven separate groups based on morphological traits. Besides, it has been reported that almond nut length and thickness, and kernel length have significant correlations with kernel length, moreover kernel width have most positive effect on kernel weight (Spiegel-Roy and Kochba, 1981; Kester *et al*., 1977).

The direct and indirect interaction of the traits which affect the yield can be used as a breeding tool, in this work we analyzed 60 local and foreign almond genotypes and cultivars to understand the casual relation among some important traits.

## 2. Materials and Methods

*Plant materials*

This research carried out as randomized complete blocks design, during the years 2013 -2014, in Horticultural sciences research institute, Karaj, Iran (51°35'E, 48°N, 1297 m above sea level, average annual max/min air temperature of 31.3/22.5°C, mean RH of 60%, the average annual precipitation of 148 mm, shallow soil pH=7.5). The average of minimum daily temperature (°C) of January to April (years 2013-2014) is given at figure 1 (Based on Iranian meteorological organization data).

We evaluated 60 almond genotypes (Table 1) using almond descriptors (Gülcan, 1985) (Table 2). All genotypes were 7-year-old and except Tuono, Supernova, Filippo Ceo and Fragiulo, the others were self-incompatible.

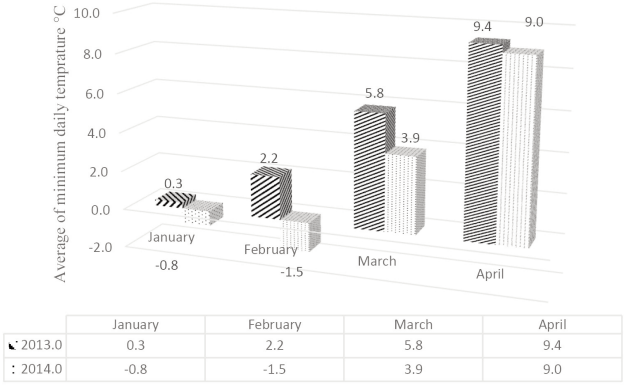



Fig. 1 - Average of minimum daily temperature (°C) from January to April (years 2013-2014).

Table 1 - Almond cultivars and genotypes evaluated in the project

| Cultivar and genotypes origin | | | | | |
|---|---|---|---|---|---|
| Foreign | | Local | | | |
| Nonpareil | Boty | k1-25 | K14-24 | K9-2 | A-200 |
| Mission | Roby | D124 | K13-40 | K9-32 | K9-20 |
| Perlice | Karmel | K8-24 | K9-24 | K6-5 | K1-16 |
| Padre | Sh-21 | Shekofeh | K8-B | K16-23 | 12-3 |
| Tuono | Sh-6 | Mamaie | K11-9 | Sahand | 8-3 |
| Filippo Ceo | Ne Plus Ultra | Sefid | K4-13 | K9-7 | D101 |
| Marcona | Sh-17 | K8-32 | K3-8 | K5-6 | |
| Supernova | K1-5 | K6-4 | K5-17 | Rabie | |
| Fragiulo | Sh-13 | K2-22 | K5-27 | D-99 | |
| Sh-12 | Sh-15 | K3-12 | K3-19 | D-8 | |
| A-230 | | Saba | Talkh asli | Z-3 | |

*Data analysis*

Descriptive statistics (minimum, maximum and mean) was used to describe and summarize the data by SPSS 22 and SAS9. Variance components, variance coefficients and Heritability were calculated by Excel 2013.

Multivariate regression analysis was performed to assess the relationship among variables by adjusted R square (ADJRSQ) procedure. Briefly, the subsets of

Table 2 - Quantitative traits using in 60 almond cultivars and genotypes

| Trait | Measuring unit | Measuring method |
|---|---|---|
| *Flower characteristics* | | |
| Flower size | cm | Caliper |
| Petal length | cm | Caliper |
| Petal width | cm | Caliper |
| Stamen number | number | Counting |
| Pistil length | cm | Caliper |
| Pistil Diameter | cm | Caliper |
| *Nut characteristics* | | |
| Nut Length | mm | Caliper |
| Nut Width | mm | Caliper |
| Nut Thickness | mm | Caliper |
| Nut Weight | g | Digital balance |
| *Kernel characteristics* | | |
| Kernel Length | mm | Caliper |
| Kernel Thickness | mm | Caliper |
| Kernel Width | mm | Caliper |
| Kernel Weight | g | Digital balance |
| Kernel Percentage | % | Calculating |
| Double kernels | % | Counting |
| *Yield* | Kg/tree | Digital balance |

the independent variables, which have the best estimation of some important dependent variables (like flower size, double kernel percentage) were selected. Then Path analysis was used to describe the direct relation among variables focusing on causality.

## 3. Results

Table 3 represents the statistical descriptive and variance component. Based on the results, the most genotypic variance (126.37 and 99.08 respectively) belonged to the double kernel and the kernel percentage, while pistil length and thickness had the least (0.01).

The most heritability belonged to kernel percentage and double kernel (92.13% and 89.74% respectively). The rest of the variables had heritability over 20.73%.

Various researches reported different almond kernel weigh heritability such as 64% (Kester *et al.,* 1977), 45% (Spiegel-Roy and Kochba, 1981) and 78% (Dicenta *et al.,* 1993 b); so to have high kernel weight (5.1 grams) progenies, the parents with high kernel weight should be chosen. In the present study kernel weigh heritability was 70.15%.

Filippo Ceo (9.6 kg/tree), k8-24 (6.84 kg/tree), Fragiulo (6.78 kg/tree) and k9-24 (6.7 kg/tree) had the most yield and Talkh asli (2.33 kg/tree), D101 (2. 44 kg/tree), 12-3 (2.45 kg/tree), 8-3 (2.8 kg kg/tree) and Sefid (2.82 kg/tree) had the lowest.

In some environmental conditions (e.g. low temperature before flowering) almond progenies have been produced double kernel nuts even if the parents did not appearance the trait (Egea and Burgos, 1995; Sánchez-Pérez *et al.,* 2007). It has been found that complexity and dominant inheritance of the mentioned trait is affected by genotype and phenotype, which have been reported by several researchers (Dicenta *et al.,* 1993 a, b; Arteaga and Socias i Company, 2001; Sánchez-Pérez *et al.*, 2007). In our study, the double kernel varied from 0 to 57%, suggests it have been influenced by the genotypes differences.

Food industries seeks for almond progenies with the medium thickness and smooth surface nuts (Sánchez-Pérez *et al.,* 2007). We found nut thickness between 9.08-20 mm. Kester *et al.* (1977) reported nut thickness heritability about 0.71, whereas we estimated it about 0.41.

In the present investigation, stepwise regression was used to identify the yield causal system which separates independent variables into direct and indirect ones (Table 4 and Fig. 2). According the data, nut weight had the most standardized beta (0. 944). As well as the nut related traits (length, width, thickness

Table 3 - Descriptive statistic, heritability, phenotypic and genotypic coefficients of almond traits

| Traits | Max | Mean | Min | Variance components | | | Heritability | Coefficient of variance | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Phenotypic | Environmental | Genotypic | | Genotypic | Phenotypic |
| Flower size | 5 | 3.88 | 2.9 | 0.39 | 0.11 | 0.28 | 71.04 | 13.57 | 16.10 |
| Petal length | 2.5 | 1.65 | 1 | 0.13 | 0.05 | 0.08 | 59.08 | 16.81 | 21.87 |
| Petal width | 1.8 | 1.35 | 0.98 | 0.08 | 0.04 | 0.04 | 52.38 | 15.54 | 21.47 |
| Stamen number | 37 | 26.83 | 15 | 25.57 | 4.00 | 21.57 | 84.35 | 17.31 | 18.84 |
| Pistil length | 1.72 | 1.46 | 1.1 | 0.03 | 0.02 | 0.01 | 45.65 | 8.10 | 11.98 |
| Pistil thickness | 0.52 | 0.26 | 0.1 | 0.04 | 0.03 | 0.01 | 23.08 | 38.21 | 79.54 |
| Nut length | 47.17 | 33.35 | 26.59 | 24.05 | 4.67 | 19.38 | 80.60 | 13.20 | 14.70 |
| Nut width | 28.82 | 20.08 | 13.28 | 15.66 | 4.00 | 11.66 | 74.45 | 17.00 | 19.70 |
| Nut thickness | 20 | 14.07 | 9.08 | 9.64 | 5.67 | 3.98 | 41.23 | 14.17 | 22.06 |
| Nut weight | 4.76 | 2.72 | 0.97 | 5.47 | 4.33 | 1.13 | 20.73 | 38.99 | 85.65 |
| Kernel length | 30.89 | 24.29 | 18 | 8.50 | 1.00 | 7.50 | 88.23 | 11.27 | 12.00 |
| Kernel width | 16.78 | 12.10 | 9.19 | 4.94 | 1.63 | 3.31 | 66.97 | 15.03 | 18.37 |
| Kernel thickness | 9.12 | 6.98 | 4.61 | 1.38 | 0.63 | 0.74 | 54.02 | 12.35 | 16.81 |
| Kernel weight | 1.67 | 1.06 | 0.56 | 0.13 | 0.04 | 0.09 | 70.15 | 28.82 | 34.41 |
| Kernel percentage | 68.88 | 42.02 | 23.52 | 137.17 | 10.80 | 126.37 | 92.13 | 26.75 | 27.87 |
| Double Kernel | 57 | 9.95 | 1 | 110.41 | 11.33 | 99.08 | 89.74 | 100.00 | 105.56 |
| Yield | 7.4 | 5.21 | 2.62 | 2.69 | 0.95 | 1.74 | 64.72 | 25.31 | 31.46 |

and weight); the kernel variables (length, width and percentage) significantly influenced the yield (Table 4). The most direct effect related to kernel weight, nut weight, nut thickness, nut width and nut length, respectively (Fig. 2). It is recommended that these traits can be assumed as the selection criteria to improve almond commercial yield.

Also, direct and indirect variables which effect kernel weight (Table 5), double kernel and flower size have been evaluated via multivariate linear regression. Nut characteristics (nut weight and width) had more regression coefficients. Kernel weight estimation entering three independent variables (for example, kernel length, width and thickness) had more $R^2$ than two variables (like kernel length and width).

Double kernel estimation using nut traits did not have acceptable $R^2$, neither kernel traits. Although the flower size was influenced by the number of stamens, petal length, and width significantly, but the $R^2$ was very low (0.353).

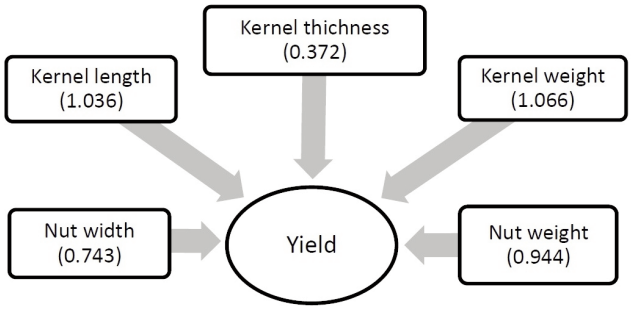



Fig. 2 - Path analysis for traits that affect almonds yield (beta coefficients have given at parenthesis).

## 4. Discussion and Conclusions

Yield improvement is the most important objective of the almond breeding programs. The commercial yield of almond fruit trees (kg kernel per tree) results the interaction of self (in)compatibility, flower

Table 4 - Yield causality analysis and stepwise correlation coefficients

| | Yield | Nut weight | Kernel weight | Kernel thickness | Kernel width | Nut width |
|---|---|---|---|---|---|---|
| Nut weight | 0.488** | 1 | | | | |
| Kernel weight | 0.655** | 0.780* | 1 | | | |
| Kernel thickness | 0.565** | 0.630** | 0.697** | 1 | | |
| Kernel width | 0.612** | 0.625** | 0.752** | 0.778** | 1 | |
| Nut width | 0.466** | 0.568** | 0.587** | 0.594** | 0.832** | 1 |
| Kernel length | 0.266* | 0.608** | 0.524** | 0.281* | 0.516** | 0.381** |
| Nut length | 0.266* | 0.580** | 0.489** | 0.203 | | |
| Kernel percentage | -0.570** | -0.176 | | | | |
| Flower size | 0 | | | | | |
| Pistil length | -0.088 | | | | | |
| Petal width | -0.053 | | | | | |
| Stamen number | -0.153 | | | | | |
| Pistil length | -0.110 | | | | | |
| Nut thickness | -0.022 | | | | | |
| Double Kernel | 0.140 | | | | | |

*, **, denote statistical significance at 5 and 1% levels, respectively.

Table 5 - Kernel weight estimation based on different independent variables

| Dependent variable | Independent variables entering to equation | | | | $R^2$ |
|---|---|---|---|---|---|
| Kernel weight | Nut weight | Nut width | Kernel thickness | Kernel percentage | 0.9461 |
| Kernel weight | Nut length | Nut weight | Nut width | Double Kernel | 0.9455 |
| Kernel weight | Nut weight | Nut width | Kernel percentage | | 0.9454 |
| Kernel weight | Kernel length | Kernel width | Kernel thickness | | 0.6193 |
| Kernel weight | Kernel length | Kernel thickness | | | 0.5888 |
| Kernel weight | Kernel width | Kernel thickness | | | 0.5827 |
| Kernel weight | Kernel length | Kernel width | | | 0.5759 |

buds density, fruit abscission, kernel dry weight and Kernel size (Garcia *et al.,* 1996).

Although some small kernel cultivar (such as 'Felisia') are ideal for chocolate bars and almond drops (Socias i Company and Felipe, 1999), large kernel almond are valued almost. The trait, as well as its correlated trait, varies each year.

High correlation between almond in shell weight and kernel weight (-0.82) (Sánchez-Pérez *et al.*, 2007), in shell kernel ratio and in shell weight (-0.72) and in shell kernel ratio and kernel weight (0.7) (Dicenta and García, 1993) have been reported previously.

Based on our result nut length significantly correlated with nut width (0.59) and nut weight (0.71). Moreover kernel weight showed high correlation with kernel length (0.64) and nut weight (0.69). the kernel weight influenced the yield directly and nut weight indirectly. While nut width, weight and diameter influenced nut length.

Based on the results K8-24 had relatively high nut length (33.5 mm), nut width (28.82 mm), kernel length (25.01 mm) and kernel width (7.48 mm). Fragiulo, K8-24 and Filippo Ceo had relatively high kernel weight (1.486, 1.378 and 1.373 gr respectively). K8-24 and Fragiulo had relatively high nut and kernel weight.

According to our findings, Kernel weight can be used as a selection criterion for almond breeding programs. Regression models for kernel weight estimation revealed that nut characteristics like length, width and weight had more $R^2$ (0.94) than the kernel characteristic such as weight, width and percentage ($R^2$= 0.61).

**Acknowledgements**

The research was funded by the Iranian Agricultural Research, Education and Extension Organization (AREEO. Project number: 8204-1. 2013/5/2).

**References**

ARTEAGA N., SOCIAS I COMPANY R., 2001 - *Heritability of fruit and kernel traits in almond*. - Acta Horticulturae, 591: 269-274.

DE GIORGIO D., LEO L., ZACHEO G., LAMASCESE N., 2007 - *Evaluation of 52 almond (*Prunus amygdalus *Batsch.) cultivars from the Apulia region in Southern Italy.* - J. Hort. Sci. Biotech., 82(4): 541-554.

DE GIORGIO D., POLIGNANO G.B., 2001 - *Evaluating the biodiversity of almond cultivars from a germplasm collection field in southern Italy*. - Sust. Glob. Farm, 56.

DICENTA F., GARCÍA J.E., 1993 - *Inheritance of kernel flavour in almond*. - Heredity, 70(3): 308-312.

DICENTA F., GARCÍA J.E., CARBONELL E., 1993 a - *Heritability of flowering, productivity and maturity in almond.* - J. Horti. Sci., 68: 113-120.

DICENTA F., GARCÍA J.E., CARBONELL E., 1993 b - *Heritability of fruit characters in almond.* - J. Hort. Sci., 68: 121-126.

DUVAL H., GRASSELLY C., 1994 - *Behaviour of some self-fertile almond selections in the south-east of France*. - Acta Horticulturae, 373: 69-74.

EGEA J., BURGOS L., 1995 - *Double kernelled fruits in almond (*Prunus dulcis *Mill.) as related to pre-blossom temperatures.* - Ann. Appl. Biol., 126: 163-168

GARCÍA J.E., DICENTA F., BERENGUER T., EGEA J., 1996 - *Programa de mejora del almendro del CEBAS-CSIC (Murcia)*. - Fruticultura Profesional, 81: 64-70.

GRADZIEL T.M., KESTER D.E., 1998 - *Breeding for self-fertility in California almond cultivars*. - Acta Horticulturae, 470: 109-117.

GÜLCAN R., 1985 - *Descriptor list for almond (*Prunus amygdalus*) (revised).* - FAO, IBPGR Secretariat, Rome, Italy.

KESTER D.E., ASAY R., 1975 - *Almonds,* pp. 387-419. - In: JANICK J., and J.N. MOORE (eds.). *Advances in fruit breeding.* Purdue Univ. Press, West Lafayette, Ind., USA, pp. 623.

KESTER D.E., GRADZIEL T.M., GRASSELLY C., 1991 - *Almonds (*Prunus*).* - Acta Horticulturae, 209: 701-758.

KESTER D.E., HANSCHE P.E., BERES W., ASAY R.N., 1977 - *Variance components and heritability of nut and kernel traits in almond*. - J. Amer. Soc. Hort. Sci., 102: 264-266.

LANSARI A., IEZZONI A.F., KESTER D.E., 1994 - *Morphological variation within collections of Moroccan almond clones and Mediterranean and North American cultivars.* - Euphytica, 78: 27-41.

ORTEGA E., DICENTA F., 2003 - *Inheritance of self-compatibility in almond: breeding strategies to assure self-compatibility in the progeny.* - Theor. Appl. Genet., 106: 904-911.

SÁNCHEZ-PÉREZ R., ORTEGA E., DUVAL H., MARTÍNEZ-GÓMEZ P., DICENTA F., 2007 - *Inheritance and relationships of important agronomic traits in almond*. - Euphytica, 155: 381-391.

SOCIAS I COMPANY R., FELIPE A.J., 1988 - *Self-compatibility in almond: transmission and recent advances in breeding.* - Acta Horticulturae, 224: 307-317.

SOCIAS I COMPANY R., FELIPE A.J., 1999 - *'Blanquerna', 'Cambra' y 'Felisia'. Tres nuevos cultivares autógamos de almendro.* - Inf. Técn. Econ. Agrar., 95(2): 111-117.

SPIEGEL-ROY P., KOCHBA J., 1974 - *The inheritance of bitter and double kernel characters in the almond.* - Z. Pflanzenzücht., 71: 319-329.

SPIEGEL-ROY P., KOCHBA J., 1981 - *Inheritance of nut and kernel traits in almond*. - Euphytica, 30: 161-174

VARGAS F.J., ROMERO M.A., 2001 - *Blooming time in almond progenies.* - CIHEAM, Options Méditerranéennes, 56: 29-34.

AHS
Advances in Horticultural Science

# Salicylic acid treatment of peach trees maintains nutritional quality of fruits during cold storage

**F. Razavi [1] (*), J. Hajilou [2], M.S. Aghdam [3]**

[1] *Department of Horticulture, Faculty of Agriculture, University of Zanjan, Zanjan, Iran.*

[2] *Department of Horticulture, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.*

[3] *Department of Horticultural Science, Imam Khomeini International University, Qazvin, Iran.*

*Key words:* antioxidant enzymes, postharvest, *Prunus persica* L., total phenols.

**Abstract: Peach trees were treated with salicylic acid at 0 (control) and 1.5 mM at 15 days before harvest to study the impacts of salicylic acid on nutritional quality of peach fruits at harvest and during storage at 1°C for 28 days. Total phenols, flavonoids, and ascorbic acid contents were significantly higher in salicylic acid treated peach fruits after cold storage, leading to fruits with higher DPPH• and FRAP radicals scavenging capacity. In addition, peach fruits treated with salicylic acid exhibited higher antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activity after storage at 1°C for 2-4 week, leading to fruits with higher firmness and lower weight loss. Thus, salicylic acid treatment of peach trees could increase nutritional quality of peach fruits consumption, due to its effect on increasing antioxidant molecules, with additional effect on delaying the fruit postharvest senescence by increasing the ROS scavenging enzymes activity.**



(*) **Corresponding author:**
razavi.farhang@znu.ac.ir

**Citation:**
RAZAVI F., HAJILOU J., AGHDAM M.S., 2018 - *Salicylic acid treatment of peach trees maintains nutritional quality of fruits during cold storage.* - Adv. Hort. Sci., 32(1): 33-40



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 12 September 2017
Accepted for publication 8 November 2017

## 1. Introduction

Peach (*Prunus persica* L.) is highly perishable climacteric stone fruit and is a rich source of ascorbic acid, carotenoids, and phenolics that are good sources of antioxidants (Tomas-Barberan *et al*., 2001). However, the rapid softening of fruit during storage at ambient temperature results in a short shelf-life of the commodity and reduced commercial fruit quality and consumer acceptance (Nunes, 2008). Due to its economic impact and also human health, great efforts have been done by researchers for delaying postharvest senescence of peach fruits during cold storage leading to fruits with higher sensory and nutritional quality by applying postharvest treatment such as modified and controlled atmosphere storage, heat treatment, glycine betaine, nitric oxide, brassinolide; 1-methylcyclopropene, methyl jasmonate, oxalic acid and salicylic acid (Cao *et al*.,

2010; Liu *et al*., 2015; Kang *et al*., 2016; Gao *et al*., 2016; Razavi and Hajilou, 2016; Shan *et al*., 2016; Yu *et al*., 2016).

Fruit ripening with oxidative fact is associated with reactive oxygen species (ROS) such as superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^-$) accumulation leading to membrane deterioration, lipid peroxidation and DNA mutation and ultimately economical quality and quantity losses of fruits results from deterioration of their cellular metabolism (Halliwell and Gutteridge, 1989). For overcome to oxidative stress during ripening which is crucial for delaying fruits deterioration and maintaining fruits sensory and nutritional quality, fruits cells employed an antioxidant system, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) as enzymatic and ascorbate and glutathione, tocopherols, phenolics, flavonoids, alkaloids and carotenoids as non-enzymatic antioxidants (Apel and Hirt, 2004). SOD vocalizes the first line of ROS scavenging and catalyzes the dismutation of $O_2^-$ to $H_2O_2$. Then, $H_2O_2$ is eliminated by the actions of APX and CAT. APX dismutes $H_2O_2$ by conversion of AA to DHA (Foyer and Noctor, 2005). Due to extra ROS production and or incapable antioxidant system activity, fruits may encounter oxidative damage during ripening leading to quality losses. Thus, delaying fruits ripening and maintaining fruits quality, due to its economic impact and also human health, can be results from effective antioxidant system activity in fruits during ripening, which is achievable by using of environmentally friendly technologies such as salicylic acid (SA) as safe signaling molecule (Hodges *et al*., 2004; Asghari and Aghdam, 2010; Kumar *et al*., 2014), which have potential in delaying ripening, enhancing quality and attenuating biotic and abiotic stress of fruits (Asghari and Aghdam, 2010).

According to capability of SA in using as preharvest treatment, Gimenez *et al*. (2014) reported that sweet cherry fruits treated at preharvest with SA at 0.5 mM and ASA at 1 mM exhibited higher total phenolics and total anthocyanins, as well as higher hydrophilic and lipophilic antioxidant activity at commercial harvest (Gimenez *et al*., 2014). Also, Gimenez *et al*. (2017) reported that sweet cherry fruits treated at preharvest with SA at 0.5 mM and ASA at 1 mM exhibited higher total phenolics and total anthocyanins, as well as higher hydrophilic antioxidant activity during storage at 2°C for 28 days. Also, cherry fruits treated at preharvest with SA and ASA exhibited higher antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) during storage at 2°C for 28 days. Valverde *et al*. (2015) reported that the sweet cherry fruits treated with preharvest 1 mM methyl salicylate exhibited higher total phenolics and anthocyanins content at harvest and during storage at 2°C for 28 days, leading to fruit with higher hydrophilic TAA (H-AA). Also, sweet cherry fruits treated with preharvest 1 mM methyl salicylate exhibited higher antioxidant enzymes CAT, APX and SOD activities during storage at 2°C for 28 days. Thus, salicylates treatment of sweet cherry trees enhances health boosting attributes of cherry fruits consumption, by increasing antioxidant molecules, with supernumerary impacts on delaying the sweet cherry fruits postharvest senescence by enhancing ROS scavenging enzymes activities (Gimenez *et al*., 2017). It has been suggested that the preharvest salicylates treatments would have commercial fondness with low earnings costs and with considerable profits in fruit nutritional quality (Gimenez *et al*., 2017). Moreover, postharvest treatment of peach fruit with SA at 2 mM enhanced higher levels of POD, CAT and SOD activities during cold storage as compared with control peaches, which were accompanied by lower polyphenol oxidase (PPO) activity. In addition, SA treated fruits exhibited higher firmness and radical scavenging activity (Tareen *et al*., 2012).

Then, the aim of this research was to evaluate for the first time the impacts of preharvest SA treatment of peach trees on bioactive molecules and the antioxidant enzymes SOD, CAT, and APX activities at harvest and during cold storage.

## 2. Materials and Methods

*Fruits and treatments*

The experiment was carried out on 5-year-old peach [*Prunus persica* (L.) Batsch 'Anjiry maleki'] trees grafted on GF 677 rootstock, in a commercial orchard located in the north-west Iran. The trees were spaced at 6×5 m, receiving identical cultural practices and trained to an open vase system. Twelve trees were selected for uniform size and fruit load and sprayed with SA at concentrations of 1.5 mM on whole tree and control trees receiving only water. A surfactant (Tween-20) was added to each solution as a wetting agent for maximum SA absorption, and sprays were applied at 15-day before commercial harvest. Fruit from control and SA treated trees were

harvested at commercial maturity and immediately transported to the laboratory. The fruits were selected for uniform size, color and absence of mechanical damage, and then one group was analyzed 24 h after harvest and another groups stored at 1±0.5°C and 90% RH for 28 d. At 7-day intervals, 5 fruits from each of three replications were selected, and left for a further 24 h at 20°C (shelf-life), and subjected to physicochemical analysis.

*Flesh firmness and weight loss*

At each sampling date, flesh firmness (N) was measured on the opposite sides of the fruit after peel removal using an Effegi penetrometer (Model FT 011) equipped with an 8 mm diameter probe. To determine the weight loss, five fruits for each replicates were weighed at harvest and at 7 day intervals during cold storage. Results were expressed as percentage of weight loss relative to the initial fruit weight.

*Antioxidant enzymes activity assays*

Crude extract for APX enzymes was performed by homogenizing 1 g of frozen fruits tissue with 5 mL of phosphate buffer 100 mM, pH= 7.8 containing 1% (w/v) PVP, 1 mM EDTA and 5 mM ascorbic acid. The homogenate was centrifuged at 18,000 *g* for 10 min at 4°C and the supernatant used for enzyme assay. APX activity was determined by the method of Nakano and Asada (1987) with some modification. The reaction mixture consisted of 3 mL of 50 mM potassium phosphate, pH 7.0, 0.2 mM ascorbic acid, 0.2 mM EDTA and 0.5 mL of crude extract, and the reaction was allowed to start by adding 0.5 mL of 0.5 mM $H_2O_2$. The decrease in absorbance at 290 nm was recorded spectrophotometrically for 3 min and APX activity expressed as U mg protein$^{-1}$.

Crude extract for CAT and SOD enzymes was performed by homogenizing 1 g of frozen fruits tissue with 3 mL of phosphate buffer 50 mM, pH= 7.8 containing 2% (w/v) PVP, 1 mM EDTA. The homogenate was centrifuged at 14,000 *g* for 20 min at 4°C and the resulting supernatant was used for enzyme assay. CAT activity was quantified following the method described by Zhang *et al*. (2013). The reaction mixture consisted of 50 mM phosphate buffer (pH 7), 15 mM $H_2O_2$ and 0.1 mL of crude extract in a final volume of 3 mL. Decreases in absorbance at 240 nm at intervals of 30 s were recorded spectrophotometrically. CAT activity expressed as U mg protein$^{-1}$. SOD activity was assayed according to the method described by Zhang *et al*. (2013). One unit of SOD activity was defined as the amount of enzyme that causes a 50% inhibition of nitro blue tetrazolium reduction under assay conditions and the results were expressed as U mg protein$^{-1}$. Total protein content in the enzyme extract was assayed according to the method described by Bradford (1976).

*Total phenolics, flavonoids and ascorbic acid contents*

The amount of total phenolics in extracts was determined according to the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). Gallic acid was used as a reference standard, and the total phenolic contents of extract were expressed as mg gallic acid equivalent 100 g$^{-1}$ fresh weight (FW). Total flavonoids content was determined in accordance with a protocol described by Kaijv *et al*. (2006). A calibration curve was obtained using quercetin as a standard, and the results were expressed as µmol quercetin equivalent (QE) 100 g$^{-1}$ FW. Ascorbic acid content in the fruits was measured by 2,6-dichlorophenol indophenol dye method (AOAC, 1984). For each sample, 10 g fresh fruits pulp was homogenized with 3% metaphosphoric acid solution and the mixture was made up to 100 mL. An aliquot of 10 ml was then titrated against the dye (2,6-dichlorophenol indophenol) till the pink color appeared. Ascorbic acid content was estimated from a calibration curve of L-ascorbic acid and results expressed as mg ascorbic acid equivalents 100 g$^{-1}$ FW.

*Total antioxidant activity*

The FRAP assay was carried out using TPTZ (2,4,6-tripyridyl-Striazine) solution according to the procedure described by Benzie and Strain (1999). FRAP reagent was prepared freshly by mixing 2.5 ml of solutions TPTZ (10 mM, dissolved in 40 mM HCl) and $FeCl_3$ (20 mM) in 25 ml of acetate buffer (300 mM concentration and 3.6 pH). A 50 µL of the diluted sample was added to 1.5 mL of FRAP reagent. The absorbance of the mixture was measured at 593 nm after 4 min. incubation at 37°C using a UV-visible spectrophotometer (T-60, PG Instrument UK). A calibration curve was built using a standard solution of $FeSO_4$ and the FRAP values of extract were expressed as mmol Fe(II)/g fresh weight.

The method of Dehghan and Khoshkam (2012) was used for measuring the DPPH radical scavenging ability of peach extracts. The amount of 50 µL of peach extract was allowed to react with 1.95 mL of DPPH radical solution (0.1 mM in methanol) for 30 min. The decrease in absorbance from the resulting solution (AS) was monitored at 517 nm in a UV-visible spectrophotometer (T-60, PG Instrument UK). Absorbance of the blank solution of DPPH (2 ml) was

used as an experimental control (AC). The radical scavenging activity (RSA %) of the peach fruits extracts was calculated according to the following formula:

$$RSA\ \% = \frac{100\ (Ac\text{-}As)}{Ac}$$

*Statistical analysis*

The experiment was performed using a factorial design with SA treatment and storage time as the two factors. Differences among means of data were analyzed by Duncan's test at p≤0.05 (n=3). All statistical analyses were performed with SPSS version 20.0.

## 3. Results and Discussion

As shown in figure 1, fruits weight loss increased and fruits firmness decreased during cold storage in control and treated fruits, but fruits weight loss was significantly lower (P<0.05) and fruits firmness were significantly higher (P<0.05) after storage at 1°C for 2-4 weeks in peach fruits coming from SA treated trees than in controls (Fig. 2). Cell wall degradation by cell wall hydrolases such as polygalactosidase (PG), pectin methyl esterase (PME), β-galactosidase (β-Gal) and xylanase along with cell membrane deterioration led to fruits softening that are associated with climacteric rise in ethylene production (Srivastava and Dwivedi, 2000). Zhang *et al*. (2003) reported that kiwifruit treated with acetyl salicylic acid exhibited lower ethylene biosynthesis during fruit ripening, results from lower ACC oxidase (ACO) and ACC synthase (ACS) activity. Kiwifruits during postharvest softening of at 20°C exhibited lower endogenous SA content, which was concurrent with higher LOX activity and higher ethylene production.

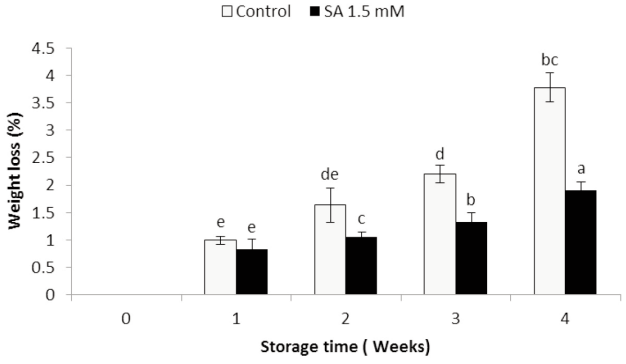



Fig. 1 - Weight loss of peach fruits treated with preharvest SA at 1.5 mM stored at 1 ± 0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

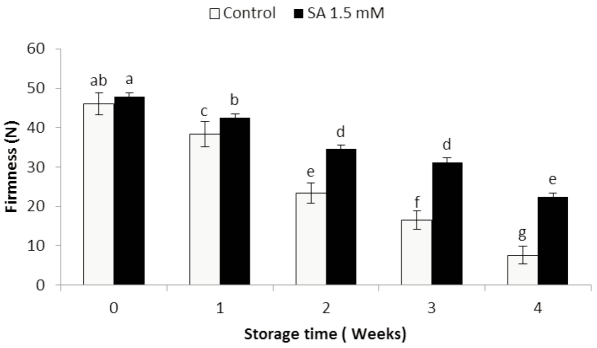



Fig. 2 - Firmness of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

LOX by generation of $O_2^-$ have a regulatory role in ethylene biosynthesis via a contribution in ACC conversion to ethylene (Xu *et al*., 2000). Kiwifruit treated with acetyl salicylic acid exhibited higher endogenous SA accumulation associated with lower LOX activity, $O_2^-$ accumulation, and ACS and ACO activities and eventually delayed ethylene biosynthesis. Zhang *et al*. (2003) suggested that the higher fruits endogenous SA accumulation leads to lower ethylene biosynthesis and higher firmness. Also, Srivastava and Dwivedi (2000) reported that SA treatment delayed banana fruits ripening, results from lower ethylene biosynthesis due to lower ACS and ACO enzymes activity. They also reported that SA treatment maintains fruits firmness, results from lower PG, xylanase and cellulase activity. Maintaining firmness in peach fruits treated with SA may be result of directly inhibition of cell wall degradation enzymes activity, indirectly decreasing ethylene production, and also higher firmness in peach fruit treated with SA could be attributed to endogenous SA accumulation which lead to lower LOX activity and ROS accumulation. Loss of weight in stored peach is mainly due to evaporation of water from the fruits and becomes apparent as shriveling. The lower weight loss in peach fruits treated with SA could be attributed to stabilization of cell membrane as well as cell wall integrity and the permeability of tissues.

Maintaining ascorbic acid content in fruits during postharvest ripening is crucial for human health, due to antioxidant function of ascorbic acid and also for human disability for ascorbic acid synthesis (Davey *et al*., 2000; Hassanpour *et al*., 2011). As shown in figure 3, ascorbic acid content decreased in control and treated fruits during storage at 1°C for 28 days, but ascorbic acid content was significantly higher during cold storage in SA treated peach fruits than in controls (P<0.05) (Fig. 3). Huang *et al*. (2008) reported

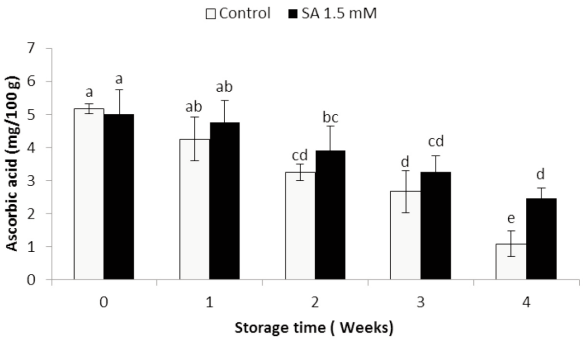



Fig. 3 - Ascorbic acid content of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

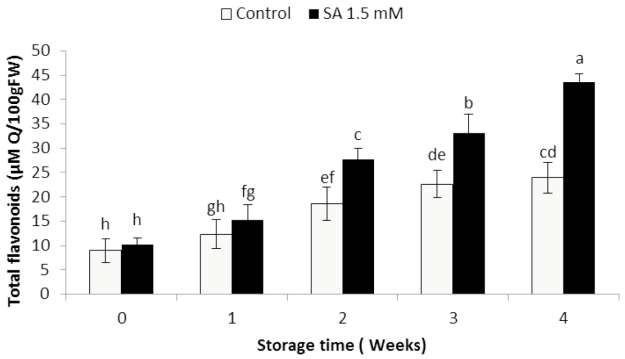



Fig. 4 - Total flavonoids content of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

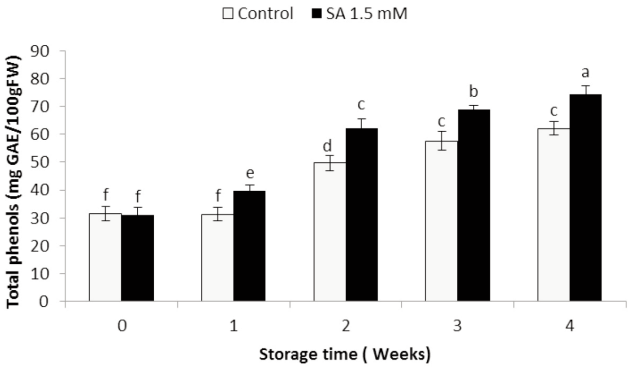



Fig. 5 - Total phenolics content of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

that navel orange fruit treated with SA exhibited higher ascorbic acid content, results from increasing cytosolic $Ca^{+2}$, enhancing GR enzyme activity, which in turn could increase GR/APX system activity leading to higher ascorbate/dehydroascorbate (AA/DHA) and glutathione/glutathione disulfide (GSH/GSSG) ratios. Also, Rao *et al*. (2011) reported that the sweet pepper treated with SA and $CaCl_2$ exhibited higher ascorbic acid content, which results from lower ascorbic acid oxidase (AAO) enzyme activity. Higher AA content in peach fruits treated with SA may be attributed to higher GR/APX system activity due to increase of cytosolic $Ca^{+2}$ concentrations and or lower AAO enzyme activity.

Enhancing phenols accumulation in fruits during postharvest ripening is crucial not only due to their contribution in nutritional quality attributes of fruits such as color, astringency, bitterness and flavor, but also phenols are superior antioxidants and display ROS scavenging activity (Hassanpour *et al*., 2011). Due to phenols ROS scavenging capacity and their function in decreasing low-density lipoproteins (LDL), consumption of fruits with higher phenols would be associated with lowered risk of heart disease (Vinson *et al*., 2001). As shown in figure 4 and 5, total phenols and flavonoids contents were significantly higher after storage at 1°C for 2-4 week in SA treated peach fruits than in controls ($P$<0.05).

It has been reported that sweet cherry fruits treated at preharvest with SA at 0.5 mM and ASA at 1 mM exhibited higher total phenolics and total anthocyanins, as well as higher total antioxidant activity at commercial harvest (Gimenez *et al*., 2014) and during storage at 2°C for 28 days (Gimenez *et al*., 2017). Also, Valverde *et al*. (2015) reported that the total phenolics and anthocyanins content were significantly higher in methyl salicylate treated sweet cherry fruits at harvest and during storage at 2°C for 28 days, leading to fruits with higher hydrophilic TAA (H-AA). Due to higher PAL enzyme activity in cornelian cherry fruits treated with SA and $CaCl_2$, which was associated with higher total phenols, flavonoids and anthocyanins accumulation (Aghdam *et al*., 2013; Dokhanieh *et al*., 2013), it can be postulated that the higher total phenols and flavonoids contents in SA treated peach fruits may attributed to higher PAL activity. Wang *et al*. (2015) reported that the apricot fruits treated with SA exhibited higher total phenols and flavonoids accumulation and higher hydrophilic antioxidant capacity. Higher hydrophilic antioxidant capacity in apricot fruits treated with SA was associated with higher PAL enzyme activity. Also, apricot fruits treated with SA exhibited higher SOD enzyme activity and lower CAT and APX enzymes activity, which leads to lower $O_2^-$ and higher $H_2O_2$ accumulation. $H_2O_2$ as second messenger can activate PAL enzyme activity, as a key enzyme in phenylpropanoids pathway, and ultimately higher total phe-

nols and flavonoids accumulation (Wang *et al*., 2015). Since peach fruits treated with SA exhibited higher CAT and APX enzymes activity, higher total phenols and flavonoids contents cannot be attributed to higher $H_2O_2$ accumulation.

As shown in figure 6 and 7, DPPH and FRAP scavenging capacity of the peach fruits treated with SA were significantly enhanced during storage at 1°C for 28 days ($P$<0.05), showing that SA treatment stimulated the scavenging capacity of the peach fruits on DPPH and FRAP radicals, which may be results from higher total phenols and flavonoids accumulation (Razavi and Hajilou, 2016). Dokhanieh *et al*. (2013) and Aghdam *et al*. (2013) reported that the cornelian cherry fruits treated with SA and $CaCl_2$ exhibited higher total phenols, flavonoids, and anthocyanins accumulation results from higher PAL enzyme activity as key enzyme in phenylpropanoid pathway which is responsible for antioxidant molecules biosynthesis. We proposed that SA treatment may stimulate the accumulation of phenol, and flavonoid in the peach fruits by activating phenylpropanoid pathway. Higher DPPH and FRAP scavenging capacity of the peach fruits treated with SA may be results from higher total phenols and flavonoids accumulation due to higher PAL enzyme activity concurrent with higher ascorbic acid accumulation due to higher cytosolic $Ca^{2+}$ and or lower AAO enzyme activity.

As shown in figure 8, 9 and 10, antioxidant enzymes CAT, SOD and APX activity increased during storage at 1°C for 28 days in control and treated fruits, and SOD and APX activities were significantly higher during all the storage period at 1°C for 28 days in SA treated peach fruits than in controls (P<0.05), while concerning CAT, higher activity was observed only after 3-4 weeks of storage. Valverde *et al*. (2015) reported that sweet cherry fruits treated at preharvest with methyl salicylate exhibited higher antioxidant enzymes CAT, APX and SOD during storage at 2°C for 28 days. Gimenez *et al*. (2017) reported that sweet cherry fruits treated at preharvest with SA at 0.5 mM and ASA at 1 mM exhibited higher antioxidant enzymes CAT, APX and SOD during storage at 2°C for 28 days. Thus, salicylates treatment of peach trees enhances health boosting attributes of peach fruits consumption, by increasing antioxidant

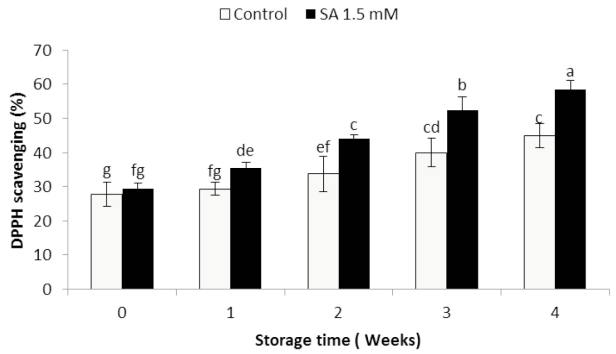



Fig. 6 - DPPH scavenging capacity of peach fruits treated with preharvest SA at 1.5 mM stored at 1 ± 0.5°C for up to 28 days. Data shown are mean±standard deviation of three replicate (n = 3).

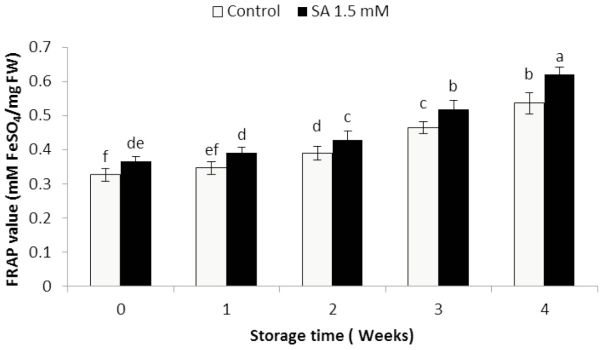



Fig. 7 - FRAP scavenging capacity of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

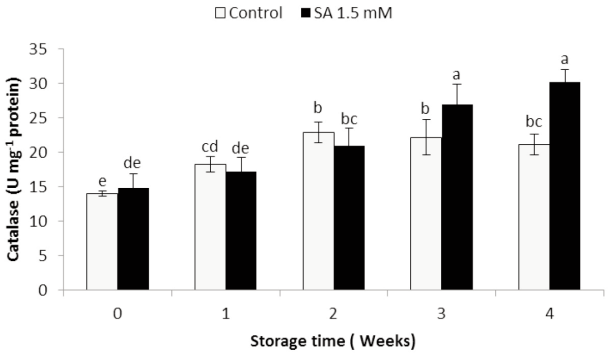



Fig. 8 - CAT activity of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

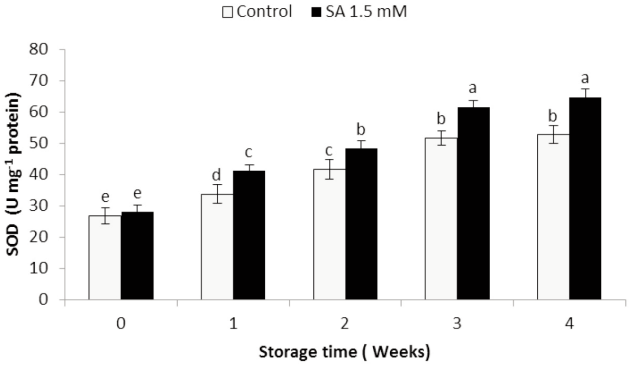



Fig. 9 - SOD activity of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

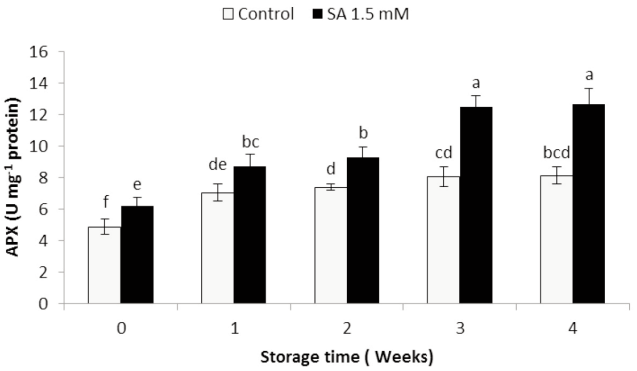



Fig. 10 - APX activity of peach fruits treated with preharvest SA at 1.5 mM stored at 1±0.5°C for up to 28 days. Data shown are mean ± standard deviation of three replicate (n = 3).

molecules, with supernumerary impacts on delaying the peach fruits postharvest senescence by enhancing ROS scavenging enzymes activities. Higher antioxidant enzymes activity, together with higher antioxidants molecules accumulation, in peach fruits during storage, as a results of preharvest SA treatment, could contribute to ROS scavenging during the postharvest ripening, which in turn, leads to delaying peach fruits postharvest ripening and senescence. SA enhance antioxidant systems activity by avoiding and/or scavenging ROS, which led to decrease oxidative stress during peach fruits ripening and ultimately maintain postharvest quality by prevention of adverse effects of ROS on fruits quality.

## 4. Conclusions

SA, as safe signaling molecule, could enhance nutritional quality and improve health promoting attributes of peach fruits consumption. In addition, the increase in antioxidant enzymes by SA preharvest treatment may result in a high ROS scavenging potential, and in turn in delaying senescence process leading to the preservation of fruits quality attributes.

## References

AGHDAM M.S., DOKHANIEH A.Y., HASSANPOUR H., REZAPOUR FARD J., 2013 - *Enhancement of antioxidant capacity of cornelian cherry (*Cornus mas*) fruit by postharvest calcium treatment*. - Sci. Hortic., 161: 160-164.

AOAC, 1984 - *Official methods of analysis. Volume 14.* - Association of Official Agricultural Chemists, Washington, DC, USA, pp. 844-847.

APEL K., HIRT H., 2004 - *Reactive oxygen species metabolism, oxidative stress, and signal transduction*. - Ann. Rev. Plant Biol., 55: 373-399.

ASGHARI M., AGHDAM M.S., 2010 - *Impact of salicylic acid on post-harvest physiology of horticultural crops*. - Trends Food Sci. Technol., 21: 502-509.

BENZIE I.F.F., STRAIN J.J., 1999 - *Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration*. - Methods Enzymol., 299: 15-27.

BRADFORD M.M., 1976 - *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. - Anal. Biochem., 72: 248-254.

CAO S., HU Z., ZHENG Y., LU B., 2010 - *Synergistic effect of heat treatment and salicylic acid on alleviating internal browning in cold-stored peach fruit*. - Postharvest Biol. Technol., 58: 93-97.

DAVEY M.D., VAN MONTAGU M., INZE D., SANMARTIN M., KANELLIS A., SMIRNOFF N., BENZIE I.J.J., STRAIN J.J., FAVELL D., FLETCHER J., 2000 - *Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability, and effects of processing*. - J. Sci. Food Agric., 80: 825-860.

DEHGHAN G., KHOSHKAM Z., 2012 - *Tin(II)-quercetin complex: Synthesis, spectral characterization and antioxidant activity*. - Food Chem., 131: 422-427.

DOKHANIEH A.Y., AGHDAM M.S., FARD J.R., HASSANPOUR H., 2013 - *Postharvest salicylic acid treatment enhances antioxidant potential of cornelian cherry fruit*. - Sci. Hortic., 154: 31-36.

FOYER C.H., NOCTOR G., 2005 - *Oxidant and antioxidant signalling in plants: a reevaluation of the concept of oxidative stress in a physiological context*. - Plant Cell Environ., 28: 1056-1071.

GAO H., ZHANG Z., LV X., CHENG N., PENG B., CAO W., 2016 - *Effect of 24-epibrassinolide on chilling injury of peach fruit in relation to phenolic and proline metabolisms*. - Postharvest Biol. Technol., 111: 390-397.

GIMENEZ M.J., SERRANO M., VALVERDE J.M., MARTINEZ-ROMERO D., CASTILLO S., VALERO D., GUILLEN F., 2017 - *Preharvest salicylic acid and acetylsalicylic acid treatments preserve quality and enhance antioxidant systems during postharvest storage of sweet cherry cultivars.* - J. Sci. Food Agric., 97(4): 1220-1228.

GIMENEZ M.J., VALVERDE J.M., VALERO D., GUILLEN F., MARTINEZ-ROMERO D., SERRANO M., CASTILLO S., 2014 - *Quality and antioxidant properties on sweet cherries as affected by preharvest salicylic and acetylsalicylic acids treatments*. - Food Chem., 160: 226-232.

HALLIWELL B., GUTTERIDGE J.M.C., 1989 - *Free radicals in biology and medicine*. - 2nd ed., Oxford University Press, Oxford, USA, pp. 97-102.

HASSANPOUR H., HAMIDOGHLI Y., HAJILOU J., ADLIPOUR M., 2011 - *Antioxidant capacity and phytochemical properties of cornelian cherry (*Cornus mas *L.) genotypes in Iran*. - Sci. Hortic., 129: 459-463.

HODGES D.M., LESTER G.E., MUNRO K.D., TOIVONEN P.M.A., 2004 - *Oxidative stress: importance for postharvest quality.* - HortSci., 39: 924-929.

HUANG R., XIA R., LU Y., HU L., XU Y., 2008 - *Effect of preharvest salicylic acid spray treatment on post-harvest antioxidant in the pulp and peel of 'Cara cara' navel*or*anges (*Citrus sinensis *L. Osbeck)*. - J. Sci. Food Agric., 88: 229-236.

KAIJV M., SHENG L., CHAO C., 2006 - *Antioxidation of flavonoids of green rhizome*. - Food Sci., 27: 110-115.

KANG R., ZHANG L., JIANG L., YU M., MA R., YU Z., 2016 - *Effect of postharvest nitric oxide treatment on the proteome of peach fruit during ripening.* - Postharvest Biol. Technol., 112: 277-289.

KUMAR S., YADAV P., JAIN V., MALHOTRA S.P., 2014 - *Isozymes of antioxidative enzymes during ripening and storage of ber (*Ziziphus mauritiana *Lamk.).* - J. Food Sci. Technol., 51: 329-334.

LIU H., CAO J., JIANG W., 2015 - *Changes in phenolics and antioxidant property of peach fruit during ripening and responses to 1-methylcyclopropene*. - Postharvest Biol. Technol., 108: 111-118.

NAKANO Y., ASADA K., 1987 - *Purification of ascorbate peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical*. - Plant Cell Physiol., 28: 131-140.

NUNES M.C.N., 2008 - *Color atlas of postharvest quality of fruits and vegetables*. - Blackwell Publ., Ames, Iowa, USA, pp. 480.

RAO T.V.R., GOL N.B., SHAH K.K., 2011 - *Effect of postharvest treatments and storage temperatures on the quality and shelf life of sweet pepper (*Capsicum annum *L)*. - Sci. Hortic., 132: 18-26.

RAZAVI F., HAJILOU J., 2016 - *Enhancement of postharvest nutritional quality and antioxidant capacity of peach fruits by preharvest oxalic acid treatment*. - Sci. Hortic., 200: 95-101.

SHAN T., JIN P., ZHANG Y., HUANG Y., WANG X., ZHENG Y., 2016 - *Exogenous glycine betaine treatment enhances chilling tolerance of peach fruit during cold storage*. - Postharvest Biol. Technol., 114: 104-110.

SINGLETON V.L., ROSSI J.A., 1965 - *Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents*. - Am. J. Enol. Vit., 16: 144-158.

SRIVASTAVA M.K., DWIVEDI U.N., 2000 - *Delayed ripening of banana fruit by salicylic acid*. - Plant Sci., 158: 87-96.

TAREEN M.J., ABBASI N.A., HAFIZ I.A., 2012 - *Postharvest application of salicylic acid enhanced antioxidant enzyme activity and maintained quality of peach cv. Flordaking fruit during storage*. - Sci. Hortic., 142: 221-228.

TOMAS-BARBERAN F.A., GIL M.I., CREMIN P., WATERHOUSE A.L., HESS-PIERCE B., KADER A.A., 2001 - *HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches and plums*. - J. Agric. Food Chem., 49: 4748-4760.

VALVERDE J.M., GIMENEZ M.J., GUILLEN F., VALERO D., MARTINEZ-ROMERO D., SERRANO M., 2015 - *Methyl salicylate treatments of sweet cherry trees increase antioxidant systems in fruit at harvest and during storage*. - Postharvest Biol. Technol., 109: 106-113.

VINSON J.A., SU X., ZUBIK L., BOSE P., 2001 - *Phenol antioxidant quantity and quality in foods: fruits*. - J. Agric. Food Chem., 49: 5315-5321.

WANG Z., MA L., ZHANG X., XU L., CAO J., JIANG W., 2015 - *The effect of exogenous salicylic acid on antioxidant activity, bioactive compounds and antioxidant system in apricot fruit.* - Sci. Hortic., 181: 113-120.

XU W.P., CHEN K.S., LI F., ZHANG S.L., 2000 - *Regulation of lipoxygenase on jasmonic acid biosynthesis in ripening kiwifruit*. - Acta Phytophysiol. Sin., 26: 507-514.

YU L., LIU H., SHAO X., YU F., WEI Y., NI Z., XU F., WANG H., 2016 - *Effects of hot air and methyl jasmonate treatment on the metabolism of soluble sugars in peach fruit during cold storage*. - Postharvest Biol. Technol., 113: 8-16.

ZHANG Y., CHEN K., ZHANG S., FERGUSON I., 2003 - *The role of salicylic acid in postharvest ripening of kiwifruit.* - Postharvest Biol. Technol., 28: 67-74.

ZHANG Z., HUBER D.J., RAO J., 2013 - *Antioxidant systems of ripening avocado (*Persea americana *Mill.) fruit following treatment at the preclimacteric stage with aqueous 1-methylcyclopropene*. - Postharvest Biol. Technol., 76: 58-64.

Adv. Hort. Sci., 2018 32(1): 41-47 DOI: 10.13128/ahs-21894

AHS
Advances in Horticultural Science

# Tomato plant growth, leaf nutrient concentrations and fruit quality under nitrogen foliar applications

**M.K. Souri*, S. Dehnavard**
*Department of Horticultural Sciences, Tarbiat Modares University, Tehran, Iran*.

*Key words*: ammonium sulfate, calcium nitrate, foliar feeding, plant nutrition, urea, yield.



(*) **Corresponding author:**
mk.souri@modares.ac.ir

**Citation:**
SOURI M.K., DEHNAVARD S., 2018 - *Tomato plant growth, leaf nutrient concentrations and fruit quality under nitrogen foliar applications.* - Adv. Hort. Sci., 32(1): 41-47



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 18 October 2017
Accepted for publication 22 November 2017

**Abstract: Tomato is a typical plant that has distinct response to different nitrogen forms in hydroponic culture. In addition, it is a well known susceptible plant to ammonium nutrition in hydroponic culture. However, its response to foliar application of nitrogen sources and N-forms has not been well investigated. In the present study, the growth, productivity and fruit quality of tomato was investigated under foliar application of nitrogen from different sources. Ammonium sulfate, urea and calcium nitrate with constant concentration of 100 mM N were weekly sprayed during four months under hydroponic culture system. A water spray treatment was considered as control. The results showed that vegetative growth parameters were significantly affected by N sources in different patterns. The factors such as plant height, leaf area, number of lateral shoots and shoot fresh and dry weight, as well as leaf nitrate reductase activity was significantly reduced by foliar application of ammonium sulfate and to less extent by urea, while there was improvement of these traits by foliar application of calcium nitrate compared to control. However, ammonium sulfate treated plants had the highest leaf SPAD value and leaf N concentrations. Plant fruiting pattern was also influenced by treatments, as ammonium sulfate spray reduced the fruit yield, and fruit vitamin C content, while it increased fruit TSS and titratable acidity. The highest value of yield and vitamin C was recorded in calcium nitrate sprayed plants.**

## 1. Introduction

Application of different fertilizers play important role in agricultural production of food commodities. Supply of adequate essential nutrients can significantly improve plant growth, quality and their nutritional values (Marschner, 2011). Different sources of each nutrient element can be applied as fertilizer to meet plant's need of that special element. Generally for most nutrients, there is little difference among effects of various sources; however, regarding nitrogen there is significant different effect of N form and sources on many vegetative and reproductive traits of plants (Souri and Roemheld, 2009).

Nitrogen fertilizers have important role in improving crop productivity; however low use efficiency rate of N fertilizers threatens sustainable plant production (Souri, 2010). On market, various nitrogen sources exist for application under field and hydroponic culture. Urea, ammonium sulfate and ammonium nitrate are the main nitrogen fertilizers for soil application, while calcium nitrate and potassium nitrate are the major nitrogen sources commonly are used in hydroponic systems (Marschner, 2011). Nitrogen forms (ammonium *vs.* nitrate) can have significant effect on morphology and physiology of plants particularly under hydroponic culture (Souri and Roemheld, 2009). In addition, ammonium instead of nitrate (Smoleń and Sady, 2009; Souri 2010; Marschner, 2011) and foliar complementation (Kolota and Osinska, 1999; Dehnavard *et al.*, 2017) supply of nitrogen can significantly improve N fertilizing efficiency in cropping systems.

There may be several potential benefits of providing nitrogen to greenhouse crops via the foliage. These include: reduced nitrogen losses through denitrification and leaching, the ability to supply nitrogen when root activity is impaired e.g. in soil or water saline conditions, and luxury supply of plants with nitrogen. In cereals and some agronomic crops, late foliar application of urea generally results in higher grain protein and N content (Fageria *et al.*, 2009). The best quality parameters of plant growth and productivity of cabbage was reported when foliar versus soil application of fertilizers was applied *(*Atanasova *et al.*, 2007).

Tomato is one of the major vegetable crops that is cultivated in many parts of the world and consumed in many dishes. The application rate of N fertilizers in tomato culture is generally high (Souri and Roemheld, 2009), with N efficiency of about 30-50% (Zotarelli *et al.*, 2009; Souri, 2010). In many greenhouses due to continuous cultivation and fertilization, soil salinity level is generally much higher than the threshold. If some levels of required N could be applied on plants as foliar spray, it can improve N use efficiency with less soil salinity buildup and less environmental side effects. In literature the tomato responses to continuous foliar spray of nitrogen sources have not well been established. Therefore, the aim of this study was to evaluate response of tomato plants to foliar application of various nitrogen forms and sources under greenhouse and hydroponic culture.

## 2. Materials and Methods

*Experimental set up*

This study was conducted during 2012 under greenhouse conditions at Faculty of Agriculture, Trabiat Modares Uni., Tehran-Iran. The experiment was done in hydroponic system with four treatments and four replications arranged in completely randomized design. Tomato seeds (*Lycopersicon esculentum* var. Money Maker) were germinated in quartz sands and after germination (in four-leaf stage) two homogeneous seedlings were transferred to pots containing a mixture of cocopeat and perlite in ratio of 3:1 (v/v). One week later one of them was removed and one week later foliar treatments were applied on plants. The nutrient solution composition was prepared following Hoagland formula (Dehnavard *et al.*, 2017). Black plastic pots as replication, with a volume of nearly 12 liter were used for plants cultivation. For first two weeks after seedling transplanting, plants were supplied with one daily application of 100-200 ml of nutrient solution. Thereafter, pots were supplied two times per day with nutrient solution of a final quantity of 250-1500 mL until end of experiment. The amount of applied solution increased with plant size and reached the amount of 1.5 liter per day at full plant size.

Treatments were foliar spray of three nitrogen sources of ammonium sulfate (AS), urea, calcium nitrate (CN) and a no spray control. All three N sources were applied in constant concentration of 100 mM N (equals to 1400 mg $L^{-1}$ N). Sprays were done on weekly basis during 4 months of active growth period from 25 March (first foliar spray) until the end of July 2012. Distilled water was sprayed in control plants. Spraying treatments were done in the early morning, one hour after sun rise.

*Measurements*

During plant growth period for four months, various vegetative traits as well as fruit harvesting records were collected. The final harvest of plants was done at the end of July. Chlorophyll index was measured two times by SPAD meter (model 502 Plus, Illinois, USA), each time with 30 readings on 3 different areas for 10 randomly selected leaves per pot that the average was presented as leaf SPAD value. SPAD readings were done at the middle of experiment and before final harvest, at 10 o'clock in the morning. Plant height, number of lateral shoots,

shoots fresh and dry weight were measured at final harvest. Cumulative harvest of fruits was recorded as final yield. Plant leaf area was measured by leaf area meter and calculated as average area of a single leaf.

Fruits after harvesting were transferred to laboratory for further quality assessment. Fruit firmness was measured by penetrometer (Model Wagner) after removing fruit skin using a blade. Fruit total soluble solids (TSS), titratable acidity (TA) and pH were determined in fruit juice squeezed by a squeezer. Fruit TSS percentage was measured by a portable refractometer (Atago, Tokyo, Japan). Fruit pH was determined using a portable pH meter, and titratable acidity was determined with titration of 5 mL of fruit juice with NaOH 0.1 N until end pH of 8.1.

For determination of vitamin C (L-ascorbic acid), 50 g of fresh fruit tissue was crushed in a porcelain mortar in vicinity of 20 mL metaphosphoric acid 6%, and then the juice transferred into a 50 ml tube, then centrifuged at 4000 rpm for 10 min. Five mL of the supernatant transferred into an Erlenmeyer flask, and received 20 mL of metaphosphoric acid 3%. Then titration of the extract was done by di-chloro phenol indophenols until appearance of a rosa color, which the amount of vitamin C (mg $100^{-1}$ g FW) was calculated accordingly and based on a standard curve of L-ascorbic acid concentrations.

From each treatment and replicates 3 fruits were kept in room temperature (25±2°C) for one week, thereafter their weight loss percentage was calculated. Total nitrogen of leaves was determined using kejeldahl method and the activity of leaf nitrate reductase enzyme (NR) was determined after grinding and homogenizing of leaf materials in a mortar containing liquid nitrogen. Nitrate reductase was extracted in a buffer consisting of 100 mM HEPES (pH 7.5), 1 mM EDTA, 7mM cystein, 3% polyvinyl polypyrolidone (PVPP), 10 Mm leupeptin, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). After preparation of extracts sulfanilamide (0.5%) and N-(1-naphthyl)-ethylenediamine dihydrochloride (0.01%) in 1.5 M hydrochloric acid (HCl) were used for color development and the amount of $NO_2^-$ was determined spectrophotometrically at 540 nm and then nitrate reductase activity was calculated accordingly.

*Statistical analysis*

Excel software was used for calculating means and standard deviations and data were analyzed by SPSS software. Comparison of means was performed at 5% by Duncan's multiple range test.

## 3. Results

The results of present study showed that plant vegetative growth parameters were significantly affected by nitrogen sources. Plant height was significantly higher in calcium nitrate treated plants compared to ammonium sulfate and urea treated plants (Table 1). The significant largest area of a single leaf and the highest number of lateral shoots were recorded in those plants which were treated with calcium nitrate, while the significant lowest records were in ammonium sulfate treated plants (Table 1). SPAD value as a chlorophyll concentration index of plants were highest in ammonium sulfate treated plants (Table 1), and there was no significant effects among other treatments.

Plant shoot fresh and dry weights were significantly affected by foliar spray of nitrogen sources. The significant highest shoot fresh and dry weight was obtained from plants treated with calcium nitrate and control plants. The significant lowest shoot fresh

Table 1 - Mean values of plant height, leaf area, number of lateral shoots and leaf SPAD of tomato plants. Plants were grown in Hoagland nutrient solution for 17 weeks

| Foliar spray treatment | Plant height (m) | Leaf area ($cm^2$) | Lateral shoots (no.) | SPAD value |
|---|---|---|---|---|
| Control (d-water) | 1.98±0.16 ab | 74.95±6.2 b | 59.25±3.5 b | 37.025±1.6 b |
| Ammonium sulfate | 1.365±0.22 c | 66.60±6.0 c | 43.25±5.6 c | 46.925±3.8 a |
| Urea | 1.753±0.17 b | 71.25±5.6 bc | 51.00±9.7 bc | 39.825±1.8 b |
| Calcium nitrate | 2.225±0.22 a | 80.60±2.7 a | 70.50±5.2 a | 39.275±1.8 b |

All three N sources were applied in constant concentration of 100 mM N.
SPAD readings were done two times at the middle of experiment and before final harvest, and the average was presented.
Data are average of 4 replications ± SD. In each column means with a common letter have no significant difference at 5% of Duncan test.

and dry weight was in plants treated with ammonium sulfate and urea (Table 2). Determination of leaf nitrogen concentration (Table 2) revealed that plants treated with ammonium sulfate and urea had significantly higher amounts compared to control and calcium nitrate treated plants. Foliar spray of nitrogen sources showed significant effect on leaf nitrate reductase enzyme activity (Table 2). Nitrate reductase is the key enzyme in nitrate assimilation that its activity depends on several factors including nitrogen and nitrate status of plant tissues. The significant highest activity of this enzyme was in leaf of plants treated with calcium nitrate followed by control plants and those treated with urea. The significant lowest nitrate reductase activity was in ammonium sulfate treated plants.

Fruiting pattern of plants was also influenced by sprays of nitrogen sources and forms (Table 3 and 4). Number of fruits per plant was highest in calcium nitrate treated plants; however, they had no significant difference with control and urea treated plants. Those plants which were treated with ammonium sulfate produced significant lowest number of fruits (Table 3). The amounts of fruit yield per plant was significantly higher in calcium nitrate treated plants (Table 3), followed by control, urea and ammonium sulfate treated plants. Fruit firmness was not affected by foliar spray of nitrogen sources; however the percentage of fruit weight loss was significantly influenced by N foliar treatments. The highest weight loss, during one week keeping fruits at room temperature, was in fruits of those plants which were treat-

Table 2 - Mean values of shoot fresh and dry weight, leaf N concentration and nitrate reductase activity of tomato plants

| Foliar spray treatment | Shoot FW (g) | Shoot DW (g) | Leaf N concentration (%) | Leaf nitrate reductase activity (μ mol $NO_2$ g FW h) |
|---|---|---|---|---|
| Control (d-water) | 1581±122 ab | 13.7±1.6 a | 2.3±0.17 b | 0.71±0.1 b |
| Ammonium sulfate | 1213±59 c | 11.5±0.8 b | 3.2±0.30 a | 0.17±0.01 d |
| Urea | 1469.5±79 b | 11.4±1.14 b | 2.9±0.32 a | 0.30±0.09 c |
| Calcium nitrate | 1758.3±186 a | 14.2±1.7 a | 2.5±0.13 b | 0.90±0.06 a |

Plants were grown in Hoagland nutrient solution for 17 weeks.
All three N sources were applied in constant concentration of 100 mM N.
Data are average of 4 replications ± SD. In each column means with a common letter have no significant difference at 5% of Duncan test.

Table 3 - Mean values of fruit number, fruit yield, fruit firmness and fruit postharvest weight loss in tomato

| Foliar spray treatment | Number of fruits $plant^{-1}$ | Fruit yield (g $plant^{-1}$) | Fruit firmness (kg $cm^2$) | Fruit weight loss (%) |
|---|---|---|---|---|
| Control (d-water) | 19.7±2.7 a | 2210.7±253 b | 1.24±0.16 a | 5.15±0.83 b |
| Ammonium sulfate | 17.2±2.6 b | 1824.5±218 c | 1.32±0.10 a | 12.25±2.04 a |
| Urea | 19.0±2.2 a | 1896.0±230 c | 1.23±0.21 a | 6.35±0.79 b |
| Calcium nitrate | 20.2±2.2 a | 2994.5±306 a | 1.38±0.11 a | 5.40±1.01 b |

Plants were grown in Hoagland nutrient solution for 17 weeks.
All three N sources were applied in constant concentration of 100 mM N.
Fruit weight loss was measured after one week in room temperature of 25±°C
Data are average of 4 replications ± SD. In each column means with a common letter have no significant diff erence at 5% of Duncan test.

Table 4 - Mean values of fruit TSS, fruit TA, fruit pH, and fruit L-ascorbic acid content in tomato under different fertilization

| Foliar spray treatment | Fruit TSS (%) | Fruit TA (%) | Fruit pH | Fruit L-ascorbic acid (mg 100 g $FW^{-1}$) |
|---|---|---|---|---|
| Control (d-water) | 5.50±0.16 ab | 3.75±0.13 b | 3.68±0.1 a | 36.8±4.1 a |
| Ammonium sulfate | 5.98±0.27 a | 4.57±0.22 a | 3.95±0.3 a | 31.0±2.1 b |
| Urea | 5.48±0.17 ab | 3.85±0.21 b | 3.90±0.2 a | 33.5±2.9 ab |
| Calcium nitrate | 5.35±0.18 b | 3.60±0.18 b | 3.78±0.2 a | 37.1±3.4 a |

Plants were grown in Hoagland nutrient solution for 17 weeks.
All three N sources were applied in constant concentration of 100 mM N.
Data are average of 4 replications ± SD. In each column means with a common letter have no significant difference at 5% of Duncan test.

ed with foliar spray of ammonium sulfate (Table 3).

Fruit TSS was also highest in ammonium sulfate treated plants and the significant lowest fruit TSS was in calcium nitrate treated plants (Table 4). Similarly, fruit titrateable acidity values (TA) unchanged in urea and calcium nitrate treated plants compared to control, however sprays of ammonium sulfate resulted in significant higher amounts of fruit TA (Table 4). Fruit juice pH was not affected by foliar sprays of nitrogen sources (Table 4). The significant highest L-ascorbic acid concentration was in calcium nitrate treated plants and control plants, while the significant lowest L-ascorbic acid was in ammonium sulfate treated plants (Table 4).

## 4. Discussion and Conclusions

The results showed that many growth and productivity traits of tomato plants were significantly affected by spray of N sources. Plant biomass production and its different parameters including plant height, leaf area, number of lateral shoots, fresh and dry weight were significantly reduced by both ammonium sulfate and to less extent by urea treatments, while calcium nitrate sprays resulted in improvement of all these growth parameters compared to AS and urea treatments.

Reduction in growth parameters of tomato due to foliar application of various concentration of ammonium sulfate have been reported by Dehnavard *et al.* (2017). On the other hand, tomato is a distinct sensitive plant to ammonium nutrition particularly under hydroponic culture (Loqué and von Wirén, 2004; Souri and Roemheld, 2009). In present study, despite plants were fed by nitrate in nutrient solution; however foliar application of ammonium forms of nitrogen (ammonium sulfate and urea) resulted in reduced growth of plants. Foliar absorption of nitrogen cannot be restricted by root medium N status, as there is always plant affinity to absorb nitrogen (Marschner, 2011; Dehnavard *et al.*, 2017). Foliar spray of ammonium sulfate in concentration of 100 and 200 mM with weekly application was resulted in significant growth restriction and less biomass production of tomato plants (Dehnavard *et al.*, 2017), while sprays of 50 mM improved tomato plant growth parameters, probably due to the fact that applied ammonium concentration and corresponding absorption was not in stressful level, but rather favored better photosynthesis and plant growth. Daily foliar application of urea as the sole N source for tomato seedlings improved seedlings growth (Nicouloud and Bloom, 1996). Despite the tomato tissue concentrations of ammonium increases significantly in 12-24 hour after foliar urea application (Nicouloud and Bloom, 1996), however from various studies it seems that plants can tolerate urea sprays better than ammonium sulfate (Souri and Roemheld, 2009). Metabolism of malate and excretion of protons play important role in maintaining pH during ammonium assimilation in the shoot following ammonium sprays (Peuke *et al.*, 1998). Urea in frequent applications and higher levels may have toxicity to plants (Bowman and Paul, 1992). However, although foliar spray of urea is common in some crops, but its physiological effects varies with season, cultivar and concentration (Bowman and Paul, 1992; Fageria *et al.*, 2009). In addition, it has been reported that foliar spray of urea compared to ammonium and nitrate may has less damage to leaves (Bowman and Paul, 1992). The absorption rate of urea is generally higher than calcium nitrate and ammonium sulfate in foliar spray (Bowman and Paul, 1992; Fageria *et al.*, 2009). However, within the tissues urea breakdowns to ammonium ions that similar to ammonium uptake can result in some toxicities (but with lesser extent) and restricted plant growth traits.

SPAD values and leaf N concentration in ammonium sulfate treated plants were significantly higher compared to control and calcium nitrate sprayed plants. There are few studies reporting the effects of foliar application of nitrogen sources on vegetable crops. Urea has been mainly used in one or limited applications with no negative side effect on plant growth (Fageria *et al.*, 2009; Zhang *et al.*, 2009). Higher leaf SPAD value by ammonium sprays could be mainly due to higher chlorophyll concentration induced by restricted leaf expansion and higher N concentrations (Souri and Roemheld, 2009; Dehnavard *et al.*, 2017). The effect of foliar applications of ammonium sulfate and urea on chlorophyll readings in this study is in agreement with the results of foliar ammonium application on tomato (Dehnavard *et al.*, 2017), and urea spray on broccoli (Yildirim *et al.*, 2007) and onion (Charbaji *et al.*, 2008). Ammonium spray probably by restriction of leaf area expansion has resulted in higher leaf N concentrations. In addition, ammonium absorption can take place by many nutrient specific and unspecific transporters, which results in less plant cell control over ammonium uptake and transport within the tissues (Souri and Roemheld, 2009).

It seems that chlorophyll biosynthesis is less sensitive to ammonium rather than other leaf parameters

such as leaf cell expansion and cell division, root and shoots growth and protein biosynthesis. It has been shown that foliar application of urea or soil application of a stabilized ammonium fertilizer (ENTEC) can significantly increase N concentrations of leaves and root in carrot (Smoleń and Sady, 2009) and tomato (Souri and Roemheld, 2009) with less nitrate content. Three foliar sprays of urea with 3 days interval on onion plants showed that bulb fresh and dry weight were increased by urea levels to 5000 mg $L^{-1}$ without any damage to leaves (Charbaji, *et al.*, 2008). The best quality parameters of dry weight, total soluble sugars, vitamin C and low nitrate content in the cabbage leaves were achieved by foliar versus soil application of fertilizers *(*Atanasova *et al.*, 2007).

Foliar application of ammonium and then urea significantly reduced nitrate reductase activity of tomato leaves (Table 2). This can be a negative factor when nitrate is actively taken up by plant roots and due to low activity of this enzyme most of nitrate accumulate in vacuoles resulting in higher nitrate accumulation in leaves and probably in fruits.

Tomato plant yield was significantly reduced by ammonium sprays, while it was increased by calcium nitrate treatment. This ammonium effect could be due to restricted vegetative growth, reduced hydraulic conductance, phloem translocation and less fruit set, while calcium nitrate had no effect on fruit number but it increased the average fruit weight resulted in higher yield compared to control. Foliar nitrogen application generally increase plant yield. The maximum increase in marketable yield in cabbage, onion, and cucumber using supplementary foliar N fertilization was 20.3%, 10.8% and 7.3%, respectively (Kolata and Osinska, 1999). Foliar fertilization significantly decreased the level of cucumber leaf infestation by downy mildew disease (Kolata and Osinska, 1999).

Changes in fruit quality traits are generally observed due to foliar N applications. In this study increase in fruit TSS by ammonium spray can be due to higher chlorophyll content of leaves and higher photosynthetic rates. It is probably feasible that foliar sprays of ammonium induced stress signals leading plant to have more fruit sugars and TSS content. Guvenc *et al.* (1995) reported that foliar urea application improved some quality (Vitamin C and Titratable acidity) and growth properties of tomato. In onion plants foliar spray of urea increased N, P, K and Ca concentration of leaves that resulted in higher photosynthesis and sugar production; however, other nutrients were not affected (Charbaji *et al.*, 2008). Similar results in carrot were found by foliar application of nitrogen sources (Smoleń and Sady, 2009).

In present study, ammonium spray reduced vitamin C content of fruits. This can be due to stress conditions induced by foliar ammonium spray and less hydraulic conductance of plant tissues (Souri and Roemheld, 2009). Changes in quality parameters of tomato by foliar nitrogen sprays are in agreement with similar studies on other vegetable crops (Guvenc *et al.*, 1995; Chaurasia *et al.*, 2005; Yildirim *et al.*, 2007; Dehnavard *et al.*, 2014), which showed that increasing nitrogen application reduced the vitamin C content. In addition, foliar application of nitrogen compounds can significantly improve plants tolerate to heat stress (Zhao *et al.*, 2008). Foliar N application can improve plant growth parameters of dry weight, relative water content and nitrate reductase activity under moderate water stress particularly with drought sensitive varieties (Zhang *et al.*, 2009).

In this experiment, foliar application of ammonium sulfate and to less extent urea reduced normal plant growth and some quality traits, while calcium nitrate generally improved tomato growth and some quality factors. However, calcium nitrate can increase nitrate content of fruits that is not suitable, and therefore is not recommended in repeated applications. Spray of ammonium sulfate increased fruit TSS and titrateable acidity while it decreased vitamin C and fruit postharvest freshness. So, restricted growth through lower height and less lateral shoots in ammonium foliar sprayed plants may suggest benefits regarding labor requirement for plant pruning-training and management under greenhouse production of tomatoes. In addition, by foliar N application (particularly Ammonium sulfate and urea) less soil salinity and less nitrate accumulation occur. Nevertheless, it is revealed that tomato still show sensitivity to ammonium nutrition through the foliage similar to root ammonium nutrition via nutrient solution.

**References**

ATANASOVA E., MITOVA I., DIMITROV I., STANCHEVA I., 2007 - *Effect of different fertilizer sources on the quality of head cabbage.* - J. Appl. Hort., 9(1): 74-76.

BOWMAN D.C., PAUL J.L., 1992 - *Foliar absorption of urea, ammonium, and nitrate by perennial ryegrass turf*. - J. Amer. Soc. Hort. Sci*.,* 117: 75-79.

CHARBAJI T., ARABI M.I.E., JAWHAR M., 2008 - *Urea foliar fertilization affects onion weight and nutrient content.* - Inter. J. Vegetable Sci., 14(3): 198-204.

CHAURASIA S.N.S., SINGH K.P., RSI M., 2005 - *Effect of foliar application of water soluble fertilizers on growth, yield, and quality of tomato (*Lycopersicon esculentum *L.)*. - Sri Lankan J. Agric. Sci., 42: 66-70.

DEHNAVARD S., SOURI M.K., MARDANLU S., 2014 - *Qualitative parameters of tomato fruits affected by foliar application of nitrogen in hydroponic culture*. - Iranian J. Plant and Seed, 30(2): 237-240.

DEHNAVARD S., SOURI M.K., MARDANLU S., 2017 - *Tomato growth responses to foliar application of ammonium sulfate in hydroponic culture.* - J. Plant Nutr., 40(3): 315-323.

FAGERIA N.K., BARBOSAFILHO M.P., MOREIRA A., GUIMAR C.M., 2009 - *Foliar fertilization of crop plant.* - J. Plant Nutr., 32: 1044-1064.

GUVENC I., PADEM H., ALAN R., 1995 - *Effect of foliar application of different levels of urea on yield and yield component of tomatoes*. - II. Turkey Nat. Hort. Symp., Adana, Turkey.

KOLOTA E., OSINSKA M., 1999 - *Efficiency of foliar nutrition of field vegetables grown at different nitrogen rates.* - Inter. Conf. Environ. Problems Associated with Nitrogen Fertilization of Field Grown Vegetable Crops, 563: 87-91.

LOQUÉ D., VON WIRÉN N., 2004 - *Regulatory levels for the transport of ammonium in plant roots.* - J. Exp. Bot., 55: 1293-1305.

MARSCHNER H., 2011 - *Mineral nutrition of higher plants. 3rd Edition* - Academic Press, London, UK, pp. 676.

NICOULOUD B.A.L., BLOOM A.J., 1996 - *Absorption and assimilation of foliarly applied urea in tomato*. - J. Amer. Soc. Hort. Sci., 121(6): 1117-1121.

PEUKE A.D., JESCHKE W.D., DIETZ K.J., SCHREIBER L., HARTUNG W., 1998 - *Foliar application of nitrate or ammonium as sole nitrogen supply in* Ricinus communis *L. Carbon and nitrogen uptake and inflows*. - New Phytol., 138(4): 675-687.

SMOLEŃ S., SADY W., 2009 - *The effect of various nitrogen fertilization and foliar nutrition regimes on the concentrations of nitrates, ammonium ions, dry matter and N-total in carrot (*Daucus carota *L.) roots.* - Scientia Hortic., 119(3): 219-231.

SOURI M.K., 2010 - *Effectiveness of chloride compared to 3, 4-dimethylpyrazole phosphate on nitrification inhibition in soil*. - Commun. Soil Sci. Plant Anal., 41(14): 1769-1778.

SOURI M.K., ROEMHELD V., 2009 - *Split daily application of ammonium cannot ameliorate ammonium toxicity in tomato plants*. - Hort. Environ. Biotechnol., 50: 384-391.

YILDIRIM E., GUVENC I., TURAN M., KARATAS A., 2007 - *Effect of foliar urea application on quality, growth, mineral uptake and yield of broccoli (*Brassica oleracea *L., var.* italica*)*. - Plant Soil and Environ., 53(3): 120-128.

ZHANG L., LI S., LIANG Z., LI S., 2009 - *Effect of foliar nitrogen application on nitrogen metabolism, water status, and plant growth in two maize cultivars under short-term moderate stress*. - J. Plant Nutr., 32(11): 1861-1881.

ZHAO W.Y., XU S., LI J.L., CUI L.J., CHEN Y.N., WANG J.Z., 2008 - *Effects of foliar application of nitrogen on the photosynthetic performance and growth of two fescue cultivars under heat stress*. - Biol. Plant., 52(1): 113-116.

ZOTARELLI L., SCHOLBERG J.M., DUKES M.D., MUÑOZ-CARPENA R., ICERMAN J., 2009 - *Tomato yield, biomass accumulation, root distribution and irrigation water use efficiency on a sandy soil, as affected by nitrogen rate and irrigation scheduling*. - Agr. Water Manag., 96(1): 23-34.

Adv. Hort. Sci., 2018 32(1): 49-59 DOI: 10.13128/ahs-21360

AHS
Advances in Horticultural Science

# Partial root-zone irrigation effects on growth, metabolism and calcium status of Mangosteen seedling (*Garcinia mangostana* L.)

**D.P. Hapsari, R. Poerwanto(*), D. Sopandie, E. Santosa**
*Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia.*

*Key words*: drought, low soil moisture, photosynthetic rate, tropical plant, water management.



(*) **Corresponding author:**
roedhy8@yahoo.co.id

**Citation:**
HAPSARI D.P., POERWANTO R., SOPANDIE D., SANTOSA E., 2018 - *Partial root-zone irrigation effects on growth, metabolism and calcium status of Mangosteeen seedling (Garcinia mangostana L.).* - Adv. Hort. Sci., 32(1): 49-59



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 18 October 2017
Accepted for publication 13 December 2017

**Abstract: Efficient irrigation technique for mangosteen seedling was evaluated, from October 2016 to May 2017, in order to determine the growth and morphophysiology of both the conventional deficit irrigation (CD) and partial root-zone irrigation (PR). A set of randomized block design, with 4 replicates each, was applied on 100% field capacity (control), 50% field capacity (CD1), 30% field capacity (CD2), and ratios of 100:50% field capacity (PR1), 100%:30% field capacity (PR2) and 50:30% field capacity (PR3). The results showed a restriction in mangosteen growth, except control, as indicated by decrease in total dry mass, which due to decrement in leaf number, photosynthetic rate and root growth. Malondialdehyde (MDA) level and glutathione peroxidase (GPX) activity was higher while proline accumulation was lower in PR compared to those of both CD and control treatments. Secondary metabolites content changes in treatments, such as octacosane, cysteamine sulfonic acid, propyl oleate, 1-nanodecene, and 2-butyn-1-ol-4metoxy were synthesized in the low soil moisture conditions. Leaf Ca-pectate, Ca-phosphate and dissolved Ca tended to increase in low soil moisture. The PR1 treated plant had the highest water use efficiency. Therefore, PR technique could be applied when the soil moisture level represents 50% (or more) of the field capacity.**

## 1. Introduction

Mangosteen (*Garcinia mangostana* L.) is a tropical perennial crop that plays an ecologically important role in agroforestry system (Wijayanto and Hartoyo, 2015). It produces an exotic fruit with high antioxidant level (Kurniawati *et al.,* 2010). However, many producing countries, such Indonesia, Malaysia, Thailand and India (Osman and Milan, 2006), are facing both fluctuations in production and yellow latex matter in mangosteen fruits production (Sdoodee and Limpun-Udom, 2002; Poerwanto *et al.,* 2010). Matra *et al.* (2016) reported that mangosteen exhibits moderate genetic variation within a population. On the other hand, according to

Martias and Mansyah (2014), the variations in mangosteen quality were affected by seasonal variation, water availability, and cultivation techniques. Furthermore, Poerwanto *et al.* (2010) stated that high water fluctuation in the soil will affect the turgor pressure so that the duct secretory of yellow latex will break out and contaminate the fruit.

As other tropical fruits, mangosteen requires low soil moisture root zone to promote flowering (Paull and Nakasone, 1998). However, extended low water status might adversely affect the plant growth (Mustaha, 2012). Preventing water fluctuation on mangosteen root zone is not easily carried out due to the fact that mangosteen is usually planted in an arid area (and depend on rainfall). On the other hand, mangosteen was spread in hills area with agroforestry system, making the irrigation setting difficult. Thus, the management of irrigation becomes an important factor.

However, mangosteen is allegedly non-responsive to irrigation due to the uniqueness of its root morphology (Wiebel *et al.,* 1994). Indeed, irrigation was observed to be ineffective in decreasing the water fluctuation (Sdoodee and Chiarawipa, 2005). Besides, the root growth of mangosteen is both slow and seasonal and it grows faster before the appearance of new leaves, steadily decreases during the leaves development, and stops post-dormancy period (Hidayat, 2005). Thus, an efficient irrigation technique is needed to decrease water fluctuation. Partial root-zone irrigation (PRI), often referred to as partial root-zone drying (PRD), is a well-known irrigation method which alternately irrigates the root zone (Sepaskhah and Ahmadi, 2010). Adwirman (2006) has already applied the PRD technique on mangosteen but further studies are still needed on both physiological responses and nutrient status of the plant. In present study, the calcium status was also analyzed, either in the form of dissolved, pectate, phosphate and oxalate.

In fact, calcium is one of important mineral in mangosteen, especially in relation to yellow latex (Dorly *et al.,* 2011; Kurniadinata, 2015) and plays an important role in the mechanism of adaptation to stress condition (Liu *et al.,* 1998; Chen *et al.,* 2002), especially on signal transduction in the responses to water deficit (Hong-Bo *et al.,* 2008). Its status was analyzed in the present research to determine the correlation between the plant root water status and the occurrence of yellow latex, especially Ca-complex such as Ca-oxalate (Korth *et al.,* 2006; Setyaningrum, 2011), Ca-dissolved, phosphate, and pectate (Saure, 2005). Therefore, this study aims to: (1) determine the growth and morpho-physiological responses of mangosteen seedlings, (2) determine the role of calcium, and (3) evaluate the level of secondary metabolites in different water status conditions.

## 2. Materials and Methods

*Orchard and plants*

Two-years old mangosteen seedlings at averages height of 30±2.02 cm and leaves number, ranged from 17 to 20, were planted on *Pasir Kuda* experimental field (±260 m asl), Bogor, Indonesia between October 2016 and May 2017. A set of randomized block design, with 4 replicates, was used for field capacities of both conventional deficit irrigation (CD) and partial root-zone irrigation (PR) methods, comprised of 100% (control), 50% (CD1), 30% (CD2), 100% A: 50% B (PR1), 100% A: 30% B (PR2) and 50% A: 30% B (PR3). Mangosteen seedlings were planted in root-boxes (50 x 40 x 20 cm) in accordance with each treatment. One side of the root-box was made of glass and covered with a black thick cloth to observe the root growth, in a non-destructive way. The root-box was divided into two parts (Fig. 1) in the PR treatment and the partitions were layered by hydrophobic plastic material, in order to avoid water flow from one side to the other side. Both sides of the root-box were filled with soil and compost at a ratio of 1:1 (w/w). Mangosteen roots were carefully cleaned and divided into two symmetrical parts and planted on both side A and side B of the root-box, respectively. The planted mangosteen seedlings were acclimatized and watered within the field capacity conditions for four weeks. In the last week of the acclimatization period 100 g dolomite [$CaMg(CO_3)_2$; ±30% CaO] was applied in each root-box.


Fig. 1 - Root-box illustration in conventional deficit (A) and partial root-zone (B) irrigation treatment. In the PR treatment, the root was planted on 2 sides, side A and side B.

*Field capacity determination*

Soil water content (WC) and humidity (RH) were measured to quickly determine the field capacity (FC). Soil water content was determined by gravimetric method (Abdurachman *et al.,* 2006). A hundred grams of soil was weighed (FW) and heated at 105°C for 24 hours (DW) and the soil water content calculated with the following equation:

$$FC = (FW - DW) / DW \times 100\%$$

Soil RH was measured by means of a soil moisture meter (HT5213, China). Soil water content and RH were measured every 3 days for the optimization (Table 1). Water treatment was applied based on Table 1. The 100% field capacity occurred when the condition was 64.39% for the soil water content and 10 for the RH. The 50% and 30% field capacity treatments were obtained at a soil water content of 28.16% (day 13) and 20.06% (day 25), respectively. In order to simplify the water availability application, the watering syllabus was set for every 2 days, 2 weeks and 3 weeks for the 100%, 50% and 30% field capacity treatments. Soil moisture meter was installed on root-box to control the soil condition.

*Measurements*

*Leaf water potential and relative water content*. Healthy mature leave samples were taken at 7.00 A.M., placed into sealed plastic, and kept in a cooler box for further observation in laboratories. The leaves were cut in a cup and measured in a WP4 chamber and leaf water potential was measured using a WP4 *Dewpoint Potential Meter* (Decagon Devices Inc, USA). The relative water content (RWC) measurement of leaves was carried out in accordance with the leaf water potential. The samples used in the leaf water potential measurement (the fresh weight/FW measured) were soaked in the cup containing distilled water. The surface of the cup was covered with filtering paper so that the leaves do not float. Afterwards, the cup was kept in a cool storage for 24 hours, then drained and weighed to determine the turgid weight (TW). Leaves samples were dried at 70°C for 3 days and weighed to determine the dry weight (DW). The RWC was calculated by means of the following equation:

$$RWC = [(FW-DW) / (TW-DW)] \times 100$$

*Photosynthesis and transpiration rates*. Photosynthesis and transpiration rate were measured prior to treatment and 2 months post-treatment using LI-COR 6400 (LI-COR Inc, USA).

*Calcium content*. Calcium content in dissolved form was determined following the method developed by Suwwan and Poovaiah (1978), while calcium in complexed forms was measured gradually according to Chen and Uetomo (1976) procedure.

*Malondialdehyde (MDA) level*. Lipid peroxidation activity was determined from MDA content that was measured by mean of Wang *et al.* (2013) procedure. Briefly, 0.4 g leaves sample was homogenized with 10 ml TCA. Homogenate was centrifuged at 4°C for 10 min at 3000 *g*. Then, 2.5 ml supernatant was added to the reagent which is containing of 0.5% TBA and 20% TCA to be incubated at 80°C for 25 min. The absorbance was read at 440, 532 and 600 nm. MDA level was calculated with the following equation:

$$MDA = 6.45 (A532-A600) - (0.56 \times A440)$$

*Proline content*. Proline content was measured following the method developed by Bates (1973). In brief, 0.5 g leaves sample was homogenized with 3% sulfosalicylic acid. The homogenate was centrifuged at 12.000 *g* for 10 min. The supernatant was mixed with reagent which contains ninhydrin and glacial acetic acid. The mixture was incubated at 100°C for 60 min and transferred into an ice bath immediately. Afterwards, sample was extracted with 4 ml toluene and stirred in vortex. The absorbance was read at 520 nm. Proline concentration was calculated using proline standard curve.

*Glutathione peroxidase (GPX) activity.* GPX activity was analyzed following the method developed by Urbanek *et al.* (1991). In brief, leaf were extracted in phosphate buffer. The reaction mixture containing

Table 1 - Optimization of soil water content and humidity of mangosteen root zone during drought stress to determine the watering syllabus

| Variables | Period of drought stress (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 4 | 7 | 10 | 13 | 16 | 19 | 22 | 25 |
| Water content (%) | **64.39 a** | 43.06 b | 44.24b | 41.37 b | **28.16 c** | 27.43 c | 24.19 cd | 21.12 d | **20.06 d** |
| Relative humidity (1-10) | **10.00 a** | 8.68 b | 8.41 bc | 8.04 bc | **7.60 c** | 7.60 c | 5.71 d | 5.71 d | **5.53 d** |

Numbers, within the same row, followed by the same letters showed no significant differences based on DMRT at a probability level of 5%. Bold numbers indicating 100%, 50% and 30% field capacity, consecutively.

phosphate buffer (pH 7.0), EDTA, guaiacol, $H_2O_2$ and 50 l enzyme extract. The enzymatic reaction was initiated by addition of extract and the increase in absorbance recorded at 470 nm for 1 min. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 $mM^{-1}$ $cm^{-1}$).

*Secondary metabolites content.* Secondary metabolites was analyzed in Jakarta Regional Health Laboratory using GCMS on fresh mature leaves from control and the most severe (PR3) treatment.

*Plant growth and biomass*. The length and volume of the root, the fresh and dry weight of plant were observed 2 months post treatments. Plants were cleaned prior to observation. The root variable observation, in PR treatment, was carried out by merging both side A and side B. The length of root was measured from the boundary between root and main stem. The volume of root was measured based on Archimedes principle. Plant was weighed to measure the fresh weight then heated at 80°C for 72 h to determine the dry weight.

*Statistical analysis*

Data were analyzed with F test and Duncan Multiple Range Test (DMRT) at a probability level of 5% using Statistical Analysis System 9.4 (SAS 9.4M4) software.

## 3. Results

The leaves in all treatments, except control, withered on three weeks post-treatment (Fig. 2). However, the plants became fresh again after rewatered, except in CD2 and PR3 treatments. Besides, abortion and drying leaves were also observed in mangosteen seedlings. Abortion of old leaves was more severe in PR2 and PR3 treatments. In fact, stressed mangosteen leaf has a unique dried pattern. The whole leaves of the stressed mangosteen seedling did not dry entirely, indeed, it dried step by step starting from both the edge and tip of the leaf blade (Fig. 2G).

The reduction in leaves number was observed since the first month of the treatment; the decrease was more severe during the second month, especially in CD2, PR2, and PR3 treatments (average 9.1, 8.7, 7.2 leaves per plant, respectively) compared to control which had 14.9 leaves (Table 2). PR1 treatment did not indicate any severe leaves abortion. Thus, there were no significant differences in terms of leaves number between control and PR1 treatments from the beginning until the end of the treatment period (Fig. 2 and Table 2).

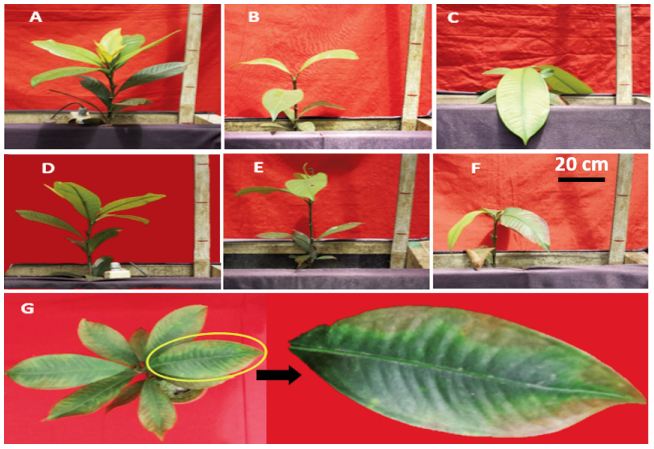



Fig. 2 - Mangosteen seedling canopy in different water availability treatments (2 months post treatment). A= control, B= conventional deficit irrigation, 50%, C =conventional deficit irrigation, 30%, D= partial root-zone irrigation 100% side A: 50% side B, E= partial root-zone irrigation 100% side A: 30% side B, F= partial root-zone irrigation 50% side A: 30% side B, G= dried pattern on mangosteen leaf).

Table 2 - Decreasing in mangosteen leaves number in different water availability treatments

| Field capacity | Number of leaves | | |
|---|---|---|---|
| | 0 MAT | 1 MAT | 2 MAT |
| Control (100%) | 18.2 | 16.6 a | 14.9 a |
| CD1 (50%) | 18.2 | 15.2 abc | 10.8 b |
| CD2 (30%) | 19.2 | 14.7 bc | 9.1 c |
| PR1 (100%A:50%B) | 19 | 16.0 ab | 13.6 a |
| PR2 (100%A:30%B) | 18.3 | 14.0 c | 8.7 c |
| PR3 (50%A:30%B) | 18.4 | 14.6 bc | 7.2 d |
| F-test | NS | * | * |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%. MAT= month after treatment.

Mangosteen leaf water potential ranged from -2.95 to -3.59 MPa before the treatments (Table 3). In present research, the leaf water potential above -3.59 MPa in two months after treatment was classified as a stress condition for seedlings, coincide with the leaf morphological characteristics which showed withered condition (Fig. 2). Leaf water content decreased post-drought treatment in both CD and PR conditions (Table 3). The lowest water potential was observed in PR3 treatment (-4.72 MPa) which had a field capacity of 50% on side A and 30% on side B. On the other hand, leaf water content also decreased in

all treatment compared to control, although the decreasing was not significant (α 5%), except for PR3 treatment which had the lowest leaf water content (Table 3).

Table 3 - Water potential and content of mangosteen seedling leaf in different water availability treatments at the beginning (0 months) and 2 months post-treatment

| Treatment (FC) | Leaf water potential (Mpa) | | Leaf water content (%) | |
|---|---|---|---|---|
| | 0 MAT | 2 MAT | 0 MAT | 2 MAT |
| Control (100%) | -2.95 | -3.09 a | 81.71 | 97.57 a |
| CD1 (50%) | -2.96 | -4.00 b | 77.5 | 71.62 ab |
| CD2 (30%) | -3.00 | -3.99 b | 73.93 | 53.55 ab |
| PR1 (100%A:50%B) | -3.59 | -4.12 b | 69.51 | 56.89 ab |
| PR2 (100%A:30%B) | -3.37 | -4.51 b | 68.08 | 58.81 ab |
| PR3 (50%A:30%B) | -3.53 | -4.72 b | 68.04 | 31.68 b |
| F-test | NS | * | NS | * |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%. MAT= month after treatment.

Drought treatment decreased both photosynthesis and transpiration rates of mangosteen seedlings two months after treatment (Table 4). PR treatments showed the lowest photosynthesis and transpiration rate and, among PR treatments, PR3 recorded the worst performances in terms of photosynthesis and transpiration rate (6.06 µmol $CO_2$ $m^{-2}$ $s^{-1}$ and 0.02 Mmol $H_2O$ $m^{-2}$ $s^{-1}$, respectively).

The dry weights of canopies and roots were significantly lower in CD and PR treated plants than control (Table 5). Unexpectedly, PR1 treatment tended to have the lowest decrement in dry weights of the canopy, roots and total plant. On the other hand, the highest decrements were observed in CD2 and PR2 treatments on both canopy and total plant dry weight, although statistically similiar. Control and PR1 treatments had the longest root apparatus (Fig. 3), although they did not differ significantly (Table 6). Drought stress significantly restricted the root growth in CD1, CD2 and PR3, excepted PR1 and PR2 treatments. Meanwhile the root volume did not show any differences among treatments.

MDA content in CD1, CD2 and PR1 treatments did not differ significantly to that of the control treatment (Fig. 4). PR2 and PR3 treatments had the high-

Table 4 - Photosynthesis and transpiration rates of mangosteen seedling in different water availability treatments at the beginning (0 months) and 2 months post-treatment

| Treatment | Photosynthesis rate (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | | Transpiration rate (Mmol $H_2O$ $m^{-2}$ $s^{-1}$) | |
|---|---|---|---|---|
| | 0 MAT | 2 MAT | 0 MAT | 2 MAT |
| Control (100%) | 16.23 | 16.20 a | 0.52 | 0.21 a |
| CD1 (50%) | 15.49 | 11.76 ab | 0.49 | 0.11 ab |
| CD2 (30%) | 18.27 | 12.05 b | 0.56 | 0.05 ab |
| PR1 (100%A:50%B) | 15.51 | 8.35 cd | 0.62 | 0.07 ab |
| PR2 (100%A:30%B) | 15.56 | 10.42 bc | 0.55 | 0.05 ab |
| PR3 (50%A:30%B) | 15.33 | 6.06 d | 0.61 | 0.02 b |
| F-test | NS | * | NS | * |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%. MAT= month after treatment.

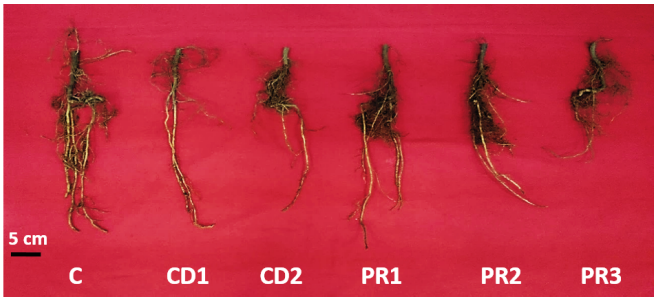



Fig. 3 - The root system of mangosteen seedling in different water availability treatments (2 months post treatment). C= control), CD1= conventional deficit irrigation 50%, CD2= conventional deficit irrigation 30%, PR1= partial root-zone irrigation 100% side A:50% side B, PR2= partial root-zone irrigation 100% side A:30% side B, PR3= partial root-zone irrigation 50% side A:30% side B.

Table 5 - Dry weight of mangosteen seedling canopy, root and total plant in different water availability treatments at 2 months post-treatment

| Treatment | Dry weight (g) | | | Relative decrease to control (%) | | |
|---|---|---|---|---|---|---|
| | Canopy | Root | Total | Canopy dry weight | Root dry weight | Total dry weight |
| Control (100%) | 12.84 a | 5.15 a | 16.43 a | - | - | - |
| CD1 (50%) | 9.20 b | 3.08 b | 12.28 b | 28.35± 0.15 | 40.19±1.02 | 39.80±0.11 |
| CD2 (30%) | 7.44 b | 3.33 b | 10.77 b | 42.06±0.11 | 35.34±1.00 | 47.21±0.10 |
| PR1 (100%A:50%B) | 9.65 b | 4.83 ab | 13.98 ab | 24.84±0.11 | 6.21±0.73 | 31.47±0.13 |
| PR2 (100%A:30%B) | 6.99 b | 3.87 ab | 10.49 b | 45.56±0.07 | 24.85±1.14 | 48.58±0.01 |
| PR3 (50%A:30%B) | 8.30 b | 3.49 b | 12.17 b | 35.36±0.08 | 32.23±2.05 | 40.34±0.03 |
| F-test | * | * | * | - | - | - |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%.

est MDA content, being 1.033 and 1.501 µmol/ml respectively, which were significantly different to that of the control treatment. Proline accumulation was in accordance with that of MDA content with the highest value in PR3 treatment followed by CD1 and CD2 (Fig. 4). Proline content in PR1 and PR2 treatments did not show any significant different to that of the control treatment. Glutathione peroxidase (GPX) activity showed different result compared to proline and MDA. In fact, the control treatment had the highest GPX activity showed not significantly differences among treatments, except PR3 treatment (Fig. 4), as a result of severe stress.

Table 6 - Root length and volume of mangosteen seedling in different water availability treatments at 2 months post-treatment

| Treatment | Root length (cm) | Root volume (ml) |
|---|---|---|
| Control (100%) | 35.00 ab | 11.67 |
| CD1 (50%) | 32.70 bc | 8.33 |
| CD2 (30%) | 29.45 cd | 6.33 |
| PR1 (100%A:50%B) | 37.75 ab | 10.33 |
| PR2 (100%A:30%B) | 33.30 abc | 7.67 |
| PR3 (50%A:30%B) | 28.00 d | 7.5 |
| F-test | * | NS |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at probability level of 5%.

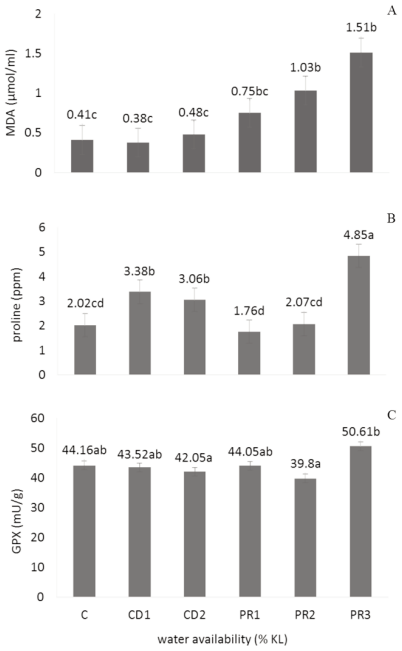



Fig. 4 - MDA (A), proline (B) content and GPX activity (C) of mangosteen seedling in different water availability treatments (2 months post-treatment). Numbers followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%.

Mangosteen seedling, on limited water condition, produced more diverse secondary metabolites than the control, especially terpenoid and fatty acid (Table 7). Five secondary metabolites were found in the leaves of control plant, i.e. oleic and hexadecenoic acid (from the fatty acid group), squalene, vitamin E and neophytadiene (from terpenoid group). Vitamin E and squalene content increased in stressed mangosteen seedling by 28% and 62% respectively, while oleic acid content decreased by 15%. On the other hand, hexadecenoic acid and neophytadiene were not detected in stress plant. There were 2 kinds of unknown compounds produced in severe stress, i.e. 1-nonadecene and 2-butyn-1-ol 4 metoxy. Dissolved Ca was mostly found in mature leaves, young leaves, roots, and branch, while Ca-pectate and Ca-phosphate were mostly found in mature leaves, roots, young leaves and branch (Table 8). Table 8 shows that both dissolved and pectate calcium contents were high in severely stressed plants, especially in young and mature leaves. Furthermore, the leaves calcium content in dissolved and pectate form of CD1 treatment were significantly higher than that of control. In CD2 treatment, calcium, in dissolved and pectate form, was tended to be lower compared to CD1 treatment but not significantly different from the control. A similar result was observed in dissolved calcium content of mangosteen seedling roots, while no differences in calcium content in branch, was observed. Table 8 also shows that Ca-phosphate and Ca-oxalate in branch were significantly higher in all PR treatments compared to control, while there were no differences in Ca-oxalate content in leaves and roots.

Table 7 - Secondary metabolites content in control and PR3 treatments of mangosteen seedling 2 months post-treatment

| Secondary metabolite compound | Group of compounds | The existence | |
|---|---|---|---|
| | | Control | PR3 |
| Oleic acid | Unsaturated fatty acid | ++ | ++ |
| Iliadic acid | Unsaturated fatty acid | - | + |
| Hexadecenoic acid | Saturated fatty acid | + | - |
| Squalene | Terpenoid | + | + |
| Vitamin E | Terpenoid | + | + |
| Neophytadiene | Terpenoid | + | - |
| Octacaine | Acyclic hydrocarbon | - | + |
| Cysteaminesulfonic acid | Amino acid | - | + |
| Propyl oleate | Ester fatty acid | - | + |
| 1-nonadecene | Unknown | - | + |
| 2-butyn-1-ol, 4 methoxy | Unknown | - | + |

- not detected, + peak area below 20%, ++ peak area between 20-50%, PR3= partial root-zone irrigation 50% side A:30% side B.

Table 8 - Calcium content of mangosteen seedling in different water availability treatments at 2 months post-treatment

| Treatment | Calcium (%) | | | | |
|---|---|---|---|---|---|
| | Dissolved | Pectate | Phosphate | Oxalate | Total |
| *Young leaves* | | | | | |
| Control (100%) | 0.133 c | 0.134 c | 0.084 c | 0.405 | 0.756 c |
| CD (50%) | 0.396 bc | 0.247 bc | 0.096 bc | 0.547 | 1.287 bc |
| CD (30%) | 0.349 c | 0.227 bc | 0.091 c | 0.428 | 1.096 bc |
| PR1 (100%A:50%B) | 0.473 bc | 0.290 abc | 0.135 ab | 0.668 | 1.568 ab |
| PR2 (100%A:30%B) | 0.826 ab | 0.429 ab | 0.135 ab | 0.372 | 1.763 ab |
| PR3 (50%A:30%B) | 1.065 a | 0.488 a | 0.157 a | 0.425 | 2.136 a |
| *Mature leaves* | | | | | |
| Control (100%) | 0.322 b | 0.267 b | 0.124 b | 0.611 | 1.326 b |
| CD (50%) | 0.741 a | 0.462 a | 0.319 a | 0.814 | 2.337 a |
| CD (30%) | 0.600 ab | 0.362 ab | 0.187 ab | 0.501 | 1.651 ab |
| PR1 (100%A:50%B) | 0.690 a | 0.428 a | 0.185 ab | 0.743 | 2.047 ab |
| PR2 (100%A:30%B) | 0.814 a | 0.346 ab | 0.209 ab | 0.68 | 2.051 ab |
| PR3 (50%A:30%B) | 0.763 a | 0.441 a | 0.174 ab | 0.473 | 1.851 ab |
| *Branch* | | | | | |
| Control (100%) | 0.078 | 0.171 | 0.086 b | 0.763 bc | 1.099 bc |
| CD (50%) | 0.134 | 0.198 | 0.107 ab | 0.923 abc | 1.364 abc |
| CD (30%) | 0.144 | 0.159 | 0.104 ab | 0.555 c | 0.963 c |
| PR1 (100%A:50%B) | 0.156 | 0.247 | 0.127 ab | 1.266 a | 1.798 a |
| PR2 (100%A:30%B) | 0.142 | 0.236 | 0.139 a | 1.108 ab | 1.627 ab |
| PR3 (50%A:30%B) | 0.148 | 0.227 | 0.123 ab | 1.335 a | 1.835 a |
| *Root* | | | | | |
| Control (100%) | 0.202 b | 0.373 | 0.148 | 0.675 | 1.399 |
| CD (50%) | 0.342 a | 0.307 | 0.219 | 0.971 | 1.84 |
| CD (30%) | 0.231 ab | 0.317 | 0.192 | 0.862 | 1.604 |
| PR1 (100%A:50%B) | 0.185 b | 0.232 | 0.167 | 1.443 | 2.052 |
| PR2 (100%A:30%B) | 0.208 b | 0.247 | 0.17 | 1.296 | 1.904 |
| PR3 (50%A:30%B) | 0.273 ab | 0.287 | 0.198 | 1.316 | 2.075 |

Numbers, in the same column, followed by the same letter indicates no significant differences based on DMRT at a probability level of 5%.

## 4. Discussion and Conclusions

In the present study, mangosteen leaves changes its morphology and aborts as a consequence of drought stress in different water treatments. Leaves abortion was more marked in CD2, PR2 and PR3 treatments, a common symptom of plants under drought stress (Munne-Bosch and Alegre, 2004). It was expected that the leaves of CD2, PR2 and PR3 treatments would accumulate more ABA and trigger the abscission process (Wingler and Roitsch, 2008; Peleg and Blumewald, 2011). On the other hand, leaves abortion in control and PR1 treatments were not significantly different from the beginning until the end of the treatment period, indicating that PR1 were not severely water stressed. Morphological changes and leaves abortion suggest that mangosteen seedlings were less tolerant to drought stress. However, mangosteen showed a low response to drought stress since the withered leaves occurred on 3 weeks post-treatment. The low response in mangosteen was expected to be a consequence of mangosteen seedling low growth as stated by Ramlan *et al.* (1992).

An high relation between leaf morphology and changes in water potential due to the variations in terms of water treatments was observed. It is expected to be the mechanism of mangosteen adjustment by decreasing water potential in tissues in order to absorb the water in the soil. Zimmerman (1978) stated that turgor potential is partially or fully maintained by osmoregulation during water stress by a reduction in the outflow of water from the cell. In previous study, the decrement of the leaf water

potential and relative water content were occurred in stressed wheat (Siddique *et al*., 2001) and *Hibiscus rosa-sinensis* (Egilla *et al.*, 2005).

The decreasing of photosynthesis and transpiration rate in stress mangosteen seedlings indicated that both moderate (50% FC) and severe (30% FC) stress conditions greatly affect mangosteen gas exchange. Purwanto and Agustono (2010) reported that photosynthesis rate in soybean, which was watered at a condition of 60% FC, decreased by 50%, while no significant fall in transpiration rate was noticed. As response to drought stress, stomata respond by reducing aperture, thereby restricting water loss, however, an inevitable consequence is the photosynthesis and canopy transpiration (Loveys *et al.*, 1999) reduction.

As mentioned in Table 6, control and PR1 treatments showed the longest roots compared to the other treatments, while the root volume did not show any differences among treatments. Hidayat (2005) reported in his research that mangosteen root has a seasonal growth where roots alternately grow with shoots. It expected lead to the slow response of mangosteen roots to drought stress. On the other hand, no significant differences were noticed in PR1 treatment in terms of root dry weight compared to control (Table 5). This was in agreement with Liu *et al.* (2006) which reported that PRD increased biomass allocation to roots. Promoting root growth under PRD has been reported in grapevine (Dry *et al.,* 2000), therefore this has been considered as an advantage of PRD irrigation.

Drought stress condition lead to high production of MDA which used as a stress indicator in the plant. High MDA content indicated that the lipid peroxidation rate, as the main effect of oxidative damage (Gill and Tuteja, 2010), was also high. Sofo *et al.* (2005) conclude that there is a direct correlation between MDA and drought stress, particularly at severe degrees of stress. In present research, the most severe case was observed in PR3 treatment which had highly MDA level that coincided with decrements in leaf water potential, photosynthesis and transpiration rates. Besides mangosteen, lipid peroxidation was also noticed in cucumber (Kubis *et al*., 2014), bean (Svetleva *et al*., 2012) and maize (Ti-da *et al*., 2006) within a stress condition.

The accumulation of proline is a common response in plants to abiotic stress. Increasing proline is a plant response to adjust its osmotic potential (Slama *et al.,* 2006) which has a strong relation with plant water potential. In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007). Present research was in agreement with Omidi (2010) which stated that in canola plants, proline content increased twofold as a result of drought stress treatment.

In the present study, the control treatment had the high GPX activity but did not show significantly differences among treatments, except PR3. It indicates that in normal condition, mangosteen seedlings have the high antioxidant activity, then became higher when the stress condition occurs, such as PR3. Halušková *et al.* (2009) stated that different abiotic stresses may cause differences in the GPX activation. Miller *et al.* (2010) noticed that GPX is plant protector against free radical. Previous studies by Sofo *et al.* (2005) and Aganchich *et al.* (2007) have reported up-regulation of the antioxidant defense system in young olive plants subjected to different degrees of water stress.

Mangosteen seedlings in limited water media produced secondary metabolites more than control, especially terpenoid and fatty acid groups. Varied antioxidant profile in different plant species is one of the principle reasons for the different adaptability and abiotic stress tolerance in plants (Jamali *et al*., 2016). Changes in composition and synthesis in drought stress condition were reported for group of terpenoid such as vitamin E (Gershenzon *et al.,* 1978), an important antioxidant that protects the cell from free radical effects (Serbinova and Packer, 1994) and photosynthetic apparatus (Fryer, 1992) from oxidative damages. In the present research, the increasing in vitamin E content by 3.2%, in stress mangosteen seedlings, was a response to the stress condition. Abiotic stress-induced changes in the fatty acid composition of plant membrane lipids mainly occur through the regulated activities of fatty acid desaturases (Upchurch, 2008). *Arabidopsis thaliana* shows remarkable tolerance to drought stress and has capacities to maintain polar lipid content and stable lipid composition, and increase the fatty acid unsaturation (Gigon *et al*., 2004).

Mangosteen seedlings under PR1 treatment had the highest calcium level. According to Bell and Biddulph (1963) some plants absorb calcium based on their physiological demand which is sometimes not comparable to the transpiration rate. As men-

tioned in Table 8, calcium contents were higher in drought stress plants in treatments of CD1, PR1, PR2 and PR3. Allegedly, there was a relationship between the response of mangosteen seedlings and both long watering interval time and calcium metabolism. CD2 treatment had a longer watering interval time compared to CD1 so that when the drought signaling occurred the water was not available, leading to a fail in calcium absorption. The increment in calcium levels in CD1, PR1, PR2 and PR3 treatments occurred in the forms of Ca-pectate and Ca-dissolved, both of which play a role in cell wall component (Peaucelle *et al.*, 2012) and cytoplasmic transduction signaling (Klimecka and Muszyńska, 2007), respectively. This was in agreement with Jin *et al.* (2016) which reported that increment in calcium levels in a salinity stress condition occurred in *Ziziphus jujuba* species. Mangosteen seedlings in PR1 treatment had higher water use efficiency (WUE) leading the possibility of calcium absorption in stress condition. In PR treatment, improvement in WUE was a results from partial stomatal closure. However, an inevitable consequence is the photosynthesis and canopy transpiration reduction (McCarthy *et al*., 2002). Hu *et al.* (2008) showed that PR method in maize able to preserve 29.5-33% water and increased WUE. The use of partial root-zone or deficit irrigation in grapevine (*Vitis vinifera*) increased WUE by about 40% while only decreasing yield by 15% when compared with full irrigation (Dos Santos *et al.,* 2003).

Mangosteen seedlings had a low response and different mechanisms in facing drought, by increasing MDA, proline, Ca-pectate and Ca-dissolved, GPX activity and synthetizing various secondary metabolites in mature leaves. In the present study, there were no significant differences between control and PR1 treatments in leaf morphology, proline, MDA and GPX activity, indicating that PR1 treatments were not severely water stressed. Therefore, present research concluded that irrigation of mangosteen through PR method was a promising method for implementation, especially in limited water areas, when the soil moisture level represents 50% (or more) of the field capacity. The implementation of PR method can reduce water requirement without significantly affecting the plant growth. It can reduce both time and labor requirements. However, improvements in the technical application to develop an effective procedure for field implementation and its relation to flowering and yellow latex of mangosteen, are still needed as further studies.

**Acknowledgements**

The authors would like to thank the Ministry of Research, Technology and Higher Education for funding and supporting the present research through PMDSU program, Batch II, in the fiscal year of 2016.

**References**

ABDURACHMAN A., HARYATI U., JUARSAH I., 2006 - *Determination of soil water content by gravimetric method*, pp. 131-142. - In: KURNIA U., F. AGUS, A. ADIMIHARDJA, and A. DARIAH (eds.) *Soil physical properties and its analysis method*. Department of Agriculture, Jakarta, Indonesia, pp 282. [*In Indonesian*].

ADWIRMAN, 2006 - *Effects of water stress on physiological and biochemical responses of mangosteen (*Garcinia mangostana *L.) plant*. - Dissertation, University Putra Malaysia, Malaysia, pp. 147.

AGANCHICH B., TAHI H., WAHBI S., ELMODAffAR C., SERRAJ R., 2007 - *Water relations, photosynthesis, growth and water use efficiency in tomato plants subjected to partial rootzone drying and regulated deficit irrigation*. - Plant Biosyst., 141: 252-264.

ASHRAF M., FOOLAD M.R., 2007 - *Roles of glycine betaine and proline in improving plant abiotic stress resistance*. - Environ. Exper. Bot., 59(2): 206-216.

BATES L.S., 1973 - *Rapid determination of free proline for water-stress studies.* - Plant and Soil, 39: 205-207.

BELL C.W., BIDDULPH O., 1963 - *Translocation of calcium: exchange versus mass flow*. - Plant Physiol., 38: 61-14.

CHEN W., PROVART N.J., GLAZEBROOK J., KATAGIRI F., CHANG H.S., EULGEM T., MAUCH F., LUAN S., ZOU G., WHITHAM S.A., BUDWORTH P.R., TAO Y., XIE Z., CHEN X., LAM S., KREPS J.A., HARPER J.F., SI-AMMOUR A., MAUCH-MANI B., HEINLEIN M., KOBAYASHI K., HOHN T., DANGL J.L., WANG X., ZHU T., 2002 - *Expression profile matrix of* Arabidopsis *transcription factor genes suggests their putative functions in response to environmental stresses*. - Plant Cell, 14: 559-574.

CHEN W.S., UETOMO S., 1976 - *Studies on calcium absorption in vegetable crops: the absorption and physiological significance of calcium in vegetative and reproductive phase of plant growth*. - J. Japan. Soc. Hort. Sci., 45: 33-42.

DORLY SOEKISMAN T., JAIME A., SILVA T., POERWANTO R., EFENDI D., FEBRIYANTI B., 2011 - *Calcium spray reduces yellow latex on mangosteen fruit (*Garcinia Mangostana *L.).* - J. Fruit Ornam. Plant Res., 19(2): 51-65.

DOS SANTOS T.P., LOPES C.M., RODRIGUES M.L., DE SOUZA C.R., MAROCO J.P., PEREIRA J.S., SILVA J.R., CHAVES M.M., 2003 - *Partial root-zone drying: effects on growth and fruit quality of field-grown grapevines* (Vitis vinifera). - Funct. Plant Biol., 30(6): 663-671.

DRY P.R., LOVEYS B.R., DURING H., 2000 - *Partial drying of the rootzone of grape. 2. Changes in the patterns of root development*. - Vitis, 39: 9-12.

EGILLA J.N., DAVIES Jr F.T., BOUTTON T.W., 2005 - *Drought stress influences leaf water content, photosynthesis, and water-use efficiency of* Hibiscus rosa-sinensis *at three potassium concentrations*. - Photosynthetica, 43: 135-140.

FRYER M.J., 1992 - *The antioxidant effects of thylakoid vitamin E (α-tocopherol).* - Plant Cell and Environ., 15: 381-392.

GERSHENZON J., LINCOLN D.E., LANGENHEIM J.H., 1978 - *The effect of moisture stress on monoterpenoid yield and composition in* Satureja douglasii. - Biochemical Systematics and Ecology, 6: 33-43.

GIGON A., MATOS A.-R., LAFFRAY D., ZUILY-FODIL Y., PHAM-THI A-T., 2004 - *Effect of drought stress on lipid metabolism in the leaves of* Arabidopsis thaliana *(Ecotype Columbia).* - Ann. Bot., 94: 345-351.

GILL S.S., TUTEJA N., 2010 - *Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants*. - Plant Phys. and Bioch., 48: 909-930.

HALUŠKOVÁ L., VALENTOVIČOVÁ K., HUTTOVÁ J., MISTRÍK I., TAMÁS L., 2009 - *Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips.* - Plant Physiol. Bioch., 47 (11-12): 1069-1074.

HIDAYAT R., 2005 - *Study of dormancy period and growth rhythm in mangoosteen (*Garcinia mangostana *L.) shoot and root*. - Bul. Agron., 33(2): 16-22. [*In Indonesian*].

HONG-BO S., LI-YE C., MING-AN S., 2008 - *Calcium as a versatile plant signal transducer under soil water stress.* - Bioessays, 30(7): 634-641.

HU T., KANG S., LI F., ZHANG J., 2008 - *Effects of partial root-zone irrigation on the nitrogen absorption and utilization of maize.* - Agric. Water Manag., 96: 208-214.

JAMALI B., ESHGHI S., KHOLDEBARIN B., 2016 - *Antioxidant responses of 'Selva' strawberry as affected by salicylic acid under salt stress.* - J. Berry Res., 6: 291-301.

JIN J., CUI H., LV X., YANG Y., WANG Y., LU W., 2016 - *Exogenous $CaCl_2$ reduces salt stress in sour jujube by reducing $Na^+$ and increasing $K^+$, $Ca^{2+}$, and $Mg^{2+}$ in different plant organs*. - J. Hortic. Sci. Biotech., 92(1): 1-9.

KLIMECKA M., MUSZYŃSKA G., 2007 - *Structure and functions of plant calcium-dependent protein kinases*. - Acta Biochimica Polonica, 54(2): 219-233.

KORTH K.L., DOEGE S.J., PARK S.H., GOGGIN F.L., WANG Q., GOMEZ S.K., LIU G., JIA L., NAKATA A.P., 2006 - Medicago truncatula *mutants demonstrate the role of plant calcium oxalate crystals as an effective defense against chewing insects.* - Plant Physiol., 141: 188-195.

KUBIS J., WIECZORE J.F., JELONEK M.A., 2014 - *Polyamines induce adaptive responses in water deficit stressed cucumber roots.* - J. Plant Res., 127: 151-158.

KURNIADINATA O.F., 2015 - *Role of calcium in overcome yellow latex in mangosteen fruit.* - Dissertation, Bogor Agricultural University, Indonesia, pp. 102. [*In Indonesian*].

KURNIAWATI A., POERWANTO R., SOBIR EFFENDI D., CAHYANA H., 2010 - *Evaluation of fruit characters, xanthones content, and antioxidant properties of various qualities of mangosteens (*Garcinia mangostana *L.).* - J. Agron. Indonesia, 38(3): 232-237.

LIU F., SHAHNAZARI A., ANDERSEN M.N., JACOBSEN S., JENSEN C.R., 2006 - *Effects of deficit irrigation (DI) and partial root drying (PRD) on gas exchange, biomass partitioning, and water use efficiency in potato*. - Scientia Horticulturae, 109(2): 113-117.

LIU Q., KASUGA M., SAKUMA Y., ABE H., MIURA S., YAMAGUCHI-SHINOZAKI K., SHINOZAKI K., 1998 - *Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low temperature responsive gene expression, respectively, in* Arabidopsis. - Plant Cell, 10: 1391-1406.

LOVEYS B.R., DRY P.R., McCARTHY M.G., 1999 - *Using plant physiology to improve the water use efficiency of horticultural crops*. - Acta Horticulturae, 537: 187-199.

MARTIAS MANSYAH E., 2014 - *Strengthening mangosteen competitiveness*, pp. 205-222. - In HARYONO, PASANDARAN E., K. SURADISASTRA, M. ARIANI, N. SUTRISNO, S. PRABAWATI, M.P. YUFDY, and A. HENDRIADI (eds.) *Strengthening competitiveness of agricultural product*. - IAARD Press, Jakarta, Indonesia, pp. 632. [I*n Indonesian*].

MATRA D.D., POERWANTO R., SANTOSA E., SOBIR HIGASHIO H., ANZAI H., INOUE E., 2016 - *Analysis of allelic diversity and genetic relationships among cultivated mangosteen* (Garcinia mangostana *L.) in Java, Indonesia using microsatellite markers and morphological characters.* - Tropical Plant Biol., 9(1): 29-41.

McCARTHY M.G., LOVEYS B.R., DRY P.R., STOLL M., 2002 - *Regulated deficit irrigation and partial root-zone drying as irrigation management techniques for grapevines*. - FAO Water Reports, 22: 79-87.

MILLER G., SUZUKI N., CIFTCI-YILMAZ S., MITTLER R., 2010 - *Reactive oxygen species homeostasis and signaling during drought and salinity stresses.* - Plant, Cell & Environ., 33(4): 453-467.

MUNNE-BOSCH S., ALEGRE L., 2004 - *Die and let live: leaf senescence contributes to plant survival under drought stress*. - Funct. Plant Biol., 31: 203-216.

MUSTAHA M.A., 2012 - *Growth improvement of mangosteen seedling by growing media modification*. - Dissertation, Bogor Agricultural University, Indonesia, pp. 223. [*In Indonesian*].

OMIDI H., 2010 - *Changes of proline content and activity of antioxidant enzymes in two canola genotype under drought stress*. - Am. J. Plant Physiol., 5 (6): 338-349.

OSMAN M.B., MILAN A.R., 2006 - *Mangosteen* - Garcinia mangostana. - Southampton Centre for Underutilised Crops, University of Southampton, Southampton, UK, pp. 170.

PAULL R.E., NAKASONE H.Y., 1998 - *Tropical fruits*. - CAB International, Wallingford, UK, pp. 445.

PEAUCELLE A., BRAYBROOK S., HÖFTE H., 2012 - *Cell wall mechanics and growth control in plants: the role of pectins revisited*. - Front Plant Sci., 3: 121.

PELEG Z., BLUMEWALD E., 2011 - *Hormone balance and abiotic stress tolerance in crop plants*. - Plant Biology, 14(3): 290-295.

POERWANTO R., DORLY MAAD M., 2010 - *Yellow latex of mangosteen.* - Proc. of Perhorti, pp. 255-260. [*In Indonesian*].

PURWANTO, AGUSTONO T., 2010 - *Study of soybean physiology in weed density variation in drought stress condition*. - J. Agroland., 17(2): 85-90.

RAMLAN M.F., MAHMUD T.M.M., HASAN B.M., KARIM M.Z., 1992 - *Studies on photosynthesis on young mangosteen plants grown under several growth conditions*. - Acta Horticulturae, 321: 482-489.

SAURE M.C., 2005 - *Calcium translocation to fleshy fruit: its mechanism and endogenous control.* - J. Hort. Sci., 17: 65-85.

SDOODEE S., CHIARAWIPA R., 2005 - *Regulating irrigation during pre-harvest to avoid the incidence of translucent flesh disorder and gamboge disorder of mangosteen fruits*. - Songklanakarin J. Sci. Technol., 27(5): 957-965.

SDOODEE S., LIMPUN-UDOM S., 2002 - *Effect of excess water on the incidence of translucent flesh disorder in mangosteen* (Garcinia mangostana L.). - Acta Horticulturae, 575: 813-820.

SEPASKHAH A.R., AHMADI S.H., 2010 - *A review on partial root-zone drying irrigation*. - Inter. J. Plant Prod., 4(4): 241-258.

SERBINOVA E.A., PACKER L., 1994 - *Antioxidant properties of α-tocopherol and α-tocotrienol.* - Methods in Enzymology, 234: 354-366.

SETYANINGRUM Y.I., 2011 - *Yellow latex secretory anatomical response, morphology and physiology of mangosteen (*Garcinia mangostana *L.) to external calcium application*. - Thesis, Bogor Agricultural University, Indonesia, pp. 61. [*In Indonesian*].

SIDDIQUE M.R.B., HAMID A., ISLAM M.S., 2001 - *Drought stress effects on water relations of wheat* - Bot. Bull. Acad. Sinica, 41: 35-39.

SLAMA I., MESSEDI D., GHNAYA T., SAVOURE A., ABDELLY C., 2006 - *Effects of water deficit on growth and proline metabolism in* Sesuvium portulacastrum. - Environ. Exper. Bot., 56: 231-238.

SOFO A., DICHIO B., XILOYANNIS C., MASIA A., 2005 - *Antioxidant defence in olive trees during drought stress: changes in activity of some antioxidant enzymes.* - Funct. Plant Biol., 32: 45-53.

SUWWAN M.A., POOVAIAH B.W., 1978 - *Association between elemental content and fruit ripening in rin and normal tomatoes.* - Plant Physiol., 13: 883-885.

SVETLEVA D., KRASTEV V., DIMOVA D., MITROVSKA Z., MITEVA D., PARVANOVA P., CHANKOVA S., 2012 - *Drought tolerance of Bulgarian common bean genotypes, characterised by some biochemical markers for oxidative stress.* - J. Central Eur. Agr., 13: 349-361.

TI-DA G., FANG-GONG S., LI-PING B., YIN-YAN L., GUANG-SHENG Z., 2006 - *Effects of water stress on the protective enzyme activities and lipid peroxidation in roots and leaves of summer maize*. - Agric. Sci., 5: 101-105.

UPCHURCH R.G., 2008 - *Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress.* - Biotechnol. Lett., 30(6): 967-977.

URBANEK H., KUZNIAK-GEBAROWSKA E., HERKA K., 1991 - *Elicitation of defense responses in bean leaves by* Botrytis cinerea *polygalacturonase*. - Acta Phys. Plant, 13: 43-50.

WANG Y., DING M., GU X., WANG J., PANG Y., GAO L., XIA T., 2013 - *Analysis of interfering substances in the measurement of malondialdehyde content in plant leaves*. - Am. J. Biochem. Biotech., 9(3): 235-242.

WIEBEL J., CHACKO E.K., DOWNTON W.J.S., LOVEYS B.S., LUDDERS P., 1994 - *Carbohydrate levels and assimilate translocation in mangosteen (*Garcinia mangostana *L.).* - Gartenbauwissenschaf, 60(2): 90-94.

WIJAYANTO N., HARTOYO A.P.P., 2015 - *Biodiversity based on agroforestry*. - Proc. Indonesia Biodiversity Community, 1(2): 242-246. [*In Indonesian*].

WINGLER A., ROITSCH T., 2008 - *Metabolic regulation of leaf senescence: interaction of sugar signaling with biotic and abiotic responses*. - Plant Biology, 10(1): 50-62.

ZIMMERMANN U., 1978 - *Physics of turgor and osmoregulation*. - Ann. Rev. Plant Physiol., 29: 121-148.

Adv. Hort. Sci., 2018 32(1): 61-69 DOI: 10.13128/ahs-21860

AHS
Advances in Horticultural Science

# Extending vase life of cut rose (*Rosa hybrida* L.) cv. Bacara by essential oils

**M.R. Salehi Salmi (*), M. Falehi Hoseini, M. Heidari, M.H. Daneshvar**
*Department of Horticulture Science, Khuzestan Ramin Agriculture and Natural Resources University, Khuzestan, Iran.*

*Key words:* antioxidant, bacteria, bent-neck, hold solutions.

**Abstract: Recent studies showed that some essential oils functions as antibacterial compounds. In this study results showed treatment with essential oils promoted vase life of cut roses via decreasing bacteria number inside the stem. We investigated components in the hydrodistilled essential oils of *Bunium persicum* Bioss, *Mentha spicata* L., *Thymus vulgaris* L. *and Satureja hortensis* L., as hold solutions, and their effects on relative fresh weight, water uptake, vase life, electrolyte leakage, anthocyanin content, soluble sugar content and number of bacteria at stem end of cut flowers of rose. GC-MS analysis of the extracted essential oil of *B. persicum*, *M. spicata*, *Th. vulgaris* and *S. hortensis* L. led to the identification of 14, 20, 13 and 14 major compounds, respectively. In cut rose, the treatment containing the essential oils extended flower opening period longer than the control. The 200 µl $l^{-1}$ essential oil of *M. spicata* treatment almost doubled the vase life of cut roses. Hence these essential oils might be powerful, environmentally friendly substitutes for the chemical compounds currently added to vase waters to control bacterial content.**



(*) **Corresponding author:**
mrsalehisalmi@gmail.com

**Citation:**
SALEHI SALMI M.R., FALEHI HOSEINI M., HEIDARI M., DANESHVAR M.H., 2018 - *Extending vase life of cut rose (Rosa hybrida L.) cv. Bacara by essential oils.* - Adv. Hort. Sci., 32(1): 61-69



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 6 October 2017
Accepted for publication 11 January 2018

## 1. Introduction

Rose (*Rosa hybrida* L.) cut flowers play an important role in the florist trade (Cairns *et al*., 2000). The cut flowers can have limited marketable value because they dehydrate during vase life as a result of decreased water uptake and the vase life is often very short. The cut flowers wilt and the floral axis becomes bent just under the flower head (bent neck). The appearance of such symptoms is considered to be caused by various factors such as bacteria, physiological responses of stems to cutting and air emboli. The development of this occlusion is correlated with the growth of bacteria at the cut surface and inside the stem (Van Doorn *et al*., 1989, 1990) and accumulation of bacteria in vase water shortens the vase life of cut rose flowers (De Witte and Van Doorn, 1988). The addition of chemicals, including some trace elements such as silver nitrate, aluminum sulphate and 8-hydroxyquinoline sulphate into holding solutions has been tried with various accomplishments in efforts to prolong the vase life of cut roses (Van Doorn *et al*., 1990).

The use of essential oils has recently become a common practice

because of strong antimicrobial activities against several pathogens. Some essential oils of plants have been reported to be effective in extending the vase life of gerbera cut flowers (Solgi *et al*., 2009). These natural organic substances have high levels of phenolic compounds such as carvacrol (Sharififar *et al*., 2007). Essential oils indicate a wide range of protective properties against disease states, oxidative stress, and microbial infection (Botelho *et al*., 2007; Yahyazadeh *et al*., 2008). And have reportedly been used for controlling plant diseases, particularly on fruits (Yahyazadeh *et al*., 2008; Ramezanian *et al*., 2016). However, there is little available information (Solgi *et al*., 2009) on the use of essential oils for the control of microbial contaminations and extending the vase life of cut flowers. Appropriate use of chemical fungicides during the postharvest could help minimize fungal infections; however, the use of chemical compounds is being discouraged for economic aims and because of growing concern about environment safety issues (Wagacha and Muthomi, 2008).

In this study, an attempt has been made to investigate the protective role of carvacrol during vase life in 'Bacara' cut-roses. This information will help to elucidate the best concentrations of these essential oils to be used in holding solutions to obtain remarkable beneficial results.

## 2. Materials and Methods

*Extraction, analysis and antioxidant activity of essential oils*

The essential oils from fresh leaves and flowers of *Bunium persicum* Bioss*, Mentha spicata* L.*, Thymus vulgaris* L. and *Satureja hortensis* L. were extracted following hydro-distillation method. The leaves and flowers (100 g) were hydrodistilled in a clevenger apparatus with 400 ml of water. The extraction was carried out for 3.5 h. The extracted oils were collected from the graduated receiver. For the deletion of water traces from the oil, the extracts were dried over anhydrous $Na_2SO_4$ and were stored in sealed ampoule bottle in a refrigerator at 4°C until for analysis.

Gas chromatographic analysis was performed with Agilent 6890A with helium as a carrier gas with a linear velocity of 30 cm/s on HP-5 column (30 m × 0.25 mm i.d, 0.25 µm film thickness). The oven was programmed to rise 50°C (3 min) isotherm, and then to 265°C at a rate of 5°C/min. Injector and detector temperatures were 300°C and 265°C, respectively. The GC mass analysis was carried out on Agilent 5975 equipped with a HP-5 column with the same characteristics as the one used in GC. Unknown essential oil was recognized by comparing its GC retention time to that of known compounds and by comparison of its mass spectra either with identified compounds or available spectra in the literature.

The antioxidant activity of essential oils was determined based on the DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical scavenging capacity by the method described by Brand-Williams *et al*. (1995). The amount of 180 µl DPPH reagent and 20 µl samples were mixed and shocked. Decline of absorbance of tested fusions was monitored for 30 min at 517 nm. For each essential oil, the tests were repeated three times.

*Plant material and general processing*

Rose flowers *(Rosa hybrida* L.) 'Bacara' grown under standard commercial conditions in a glass covered greenhouse were used as plant material. Flowers were harvested at commercial stage to 0.45 m in length. Flower stems were placed in tap water after harvest and transported by non-refrigerated car to the laboratory within 2 h. The flowers were placed randomly in 3 L glass vases containing 1 L of essential oils solutions of 100, 200 and 400 $\mu l.l^{-1}$ concentrations for 24 h. Distilled water was used as control. Sucrose (4%, w/v) was added to all solutions and the concentrations of essential oils were prepared in Tween 20 (0.1%). Then, flowers were individually placed in glass bottles of 25 cm height. Each bottle contained approximately 200 ml of distilled water. Bottles were standing in a controlled room under the following conditions: 12 h photoperiod (06.00-18.00) at photosynthetically activated radiation of 12 µmol $m^{-2}$ $s^{-1}$ provided by fluorescent lamps, 25±1°C and relative humidity of 60-70%. In total, nine floin three replications were placed in Bottles.

*Measurements*

Relative fresh weight was calculated by the formula:

$$RFW\ (\%\ initial\ fresh\ weight) = (FW_t/FW_{t=0}) \times 100$$

where RFW was Relative fresh weight, $FW_t$ was the fresh weight of flower (g) at t (in day) = 0, 1, 2, 3, etc., and $FW_t$=0 was the fresh weight of the same flower (g) at t (in day) = 0 (He *et al*., 2006).

*Total vase solution uptake.* Weight of vases containing vase solution without the cut stems were measured during the experiment period and flower opening was measured daily until the flowers

attained their largest size in the vase and began loss their size.

Cut flower longevity was documented as days vase life from the stage flowers were placed into glass bottles till the end of vase life defined as the time that flowers showed indications of petal wilting or curling, fall of one or more petals (Van der Sman *et al*., 1996).

Electrolyte leakage was measured using an electrical conductivity meter. Leaves were excised and washed with deionized water. After drying with tissue paper, 1 g fresh weight of leaves were cut into small pieces (about 1 $cm^2$) and then immersed in 20 ml deionized water and incubated at 25°C. After 24 h, electrical conductivity ($EC_1$) and after 48 h ($EC_2$) of the bathing solution were recorded for the samples (Lutts *et al*., 1996).

*Determination of anthocyanins*. Samples of petal (100 mg) were powdered in a pre-chilled pestle and extracted into methanol: HCl (99:1). Samples were incubated overnight at 4°C in darkness. The content of anthocyanins determined spectrophotometrically at 535 nm using an extinction coefficient (ε) of 29,600. The final concentration of anthocyanin was calculated based on weight of sample used and total volume of the extract.

For determination of soluble sugars, fresh petals (0.30 g) were put into test tubes with 10 mL distilled water and sealed. The tubes were incubated in a water bath at 90°C for 30 min, then the tubes were removed and the volume set at 25 ml. The amount of 0.5 ml of supernatant was mixed with 1.5 ml distilled water, 5 ml concentrated sulfuric acid and 0.5 ml anthrone. The mixed solution was read at 620 nm (Frohlich and Kutscherah, 1995).

*Ascorbate peroxidase activity*. The fresh leaves (1 g) were mixed in 100 mM potassium phosphate buffer (pH 7.8) containing 1% (w:v) PVP (polyvinylpyrrolidone), 0.1 mM EDTA (ethylenediaminetetraacetic acid), and 0.5% (v:v) Triton X-100 at 4°C, except that in the case of APX activity leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 5 mM ascorbate. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 18000 *g* for 20 min at 4°C. Peroxidase activity was assayed as per the method of Kar and Mishra (1976).

*Determination of bacterial numbers*. The lowermost 2 cm (about 0.5 g in weight) of the stems were cut. The samples were washed 3 times with sterile deionized water. They were ground and then dilution was made with a 0.9% normal salt solution. Liquid extract (0.1 ml) was spread on Plate Count Agar (PCA) plates. Before counting of bacteria, PCA plates were incubated at 38°C for 2 days (Balestra *et al*., 2005).

*Statistical analysis*

Completely randomized experiment designs were used. Statistical significance between mean values was assessed using analysis of variance (ANOVA) and a conventional Tukey's test at $p \leq 0.05$ using SAS (9.1) statistical software. For data of maximum flower opening, standard deviation (SD) was calculated and data are expressed in mean±SD of three replicates.

## 3. Results and Discussion

GC-MS analysis of the extracted essential oil of *B. persicum*, *M. spicata*, *Th. vulgaris* and *S. hortensis* L. led to the identification of 14, 20, 13 and 14 major compounds, respectively (Table 1). The results revealed that 5 major components of essential oil from *B. persicum* contained γ-Terpinene, β-Caryophyllene, Cuminyl acetate, p-Cymene and β-Pinene. Investigation is limited about the chemical composition of the *B. persicum* essential oil. It has been reported that γ-Terpinene and β-Caryophyllene are main components (Shahsavari *et al*., 2008). Five major components of *Mentha spicata* L. essential oil were Carvone, 1,8-Cineole, Borneol, Limonene and Pulegone. *Mentha* essential oil was characterized by the dominant presence of carvone (56.9%) in agreement with results from other authors, who reported the presence of 76.65% in samples collected from India (Chauhan *et al*., 2009) and 64.4% in samples collected in Montenegro (Scherer *et al*., 2013). Carvacrol (67.3%), Thymol (12.7%), α-Pinene (4.25%), γ-Terpinene (3.53%) and Eucalyptol (3.32%) were most abundant in essential oil of *Th. vulgaris*. Similar results have been obtained by Ben El Hadj Ali *et al*., (2014) with *Th. numidicus*. The principle compounds identified in essential oil of *Satureja hortensis* L. were Carvacrol (66.4%), p-Cymene (18.1%), Linalool (4.5%), γ-Terpinene (3.95%) and Borneol (1.8%). A comparison of our study with a previous report (Ghasemi-Pirbalouti *et al*., 2014) suggests that the few differences in the volatile composition of the plant material could be attributed to a series of factors such as the genotype, plant developmental stage, environmental conditions and the methods of extraction.

All assayed essential oils were able to reduce

Table 1 - Major natural volatile components in the hydrodistilled essential oils of *B. persicum*, *M. spicata*, *Th. vulgaris* and *S. hortensis*

| No. | Component | Retention indices | Percentage (%) | | | |
|---|---|---|---|---|---|---|
| | | | *B. persicum* | *M. spicata* | *Th. vulgaris* | *S. hortensis* |
| 1 | α-Thujene | 924 | 0.05 | 0.03 | 0.18 | 0.3 |
| 2 | α-Pinene | 932 | 1.75 | 1.09 | 4.25 | 0.5 |
| 3 | Camphene | 946 | - | 0.56 | - | - |
| 4 | Sabinene | 971 | 0.8 | 0.74 | - | - |
| 5 | β-Pinene | 977 | 3.68 | 1.59 | 0.44 | 0.1 |
| 6 | Myrcene | 989 | 0.71 | 0.41 | 0.86 | 0.6 |
| 7 | 3-Octanol | 922 | - | 0.21 | - | 0.1 |
| 8 | Eucalyptol | 1018 | - | - | 3.32 | - |
| 9 | p-Cymene | 1021 | 6.91 | 0.83 | - | 18.1 |
| 10 | Limonene | 1036 | 3.28 | 5.69 | 0.44 | - |
| 11 | 1,8-Cineole | 1039 | 0.1 | 13.53 | 0.2 | - |
| 12 | γ-Terpinene | 1059 | 27.61 | 0.58 | 3.53 | 3.95 |
| 13 | Linalool | 1086 | 1.1 | - | 0.71 | 4.5 |
| 14 | Menthone | 1154 | - | 0.26 | - | - |
| 15 | Borneol | 1159 | - | 8.15 | - | 1.8 |
| 16 | α-Terpineol | 1169 | 0.98 | 3.25 | 0.54 | 1.02 |
| 17 | Pulegone | 1231 | 0.2 | 3.28 | - | - |
| 18 | Carvone | 1237 | - | 56.94 | - | - |
| 19 | Thymol | 1287 | 3.4 | 0.67 | 12.79 | 0.3 |
| 20 | Carvacrol | 1293 | 0.35 | 0.47 | 67.36 | 66.46 |
| 21 | β-Caryophyllene | 1415 | 25.1 | 0.93 | - | 0.85 |
| 22 | Cuminyl acetate | 1434 | 16.68 | - | - | - |
| 23 | Caryophyllene oxide | 1580 | 0.47 | 0.16 | 1.23 | 1.2 |

DPPH, reaching 50% of reduction with $IC_{50}$ values ranging 54.19±0.87 mg/mL for *M. spicata*, to 258.16 ± 1.53 mg/mL for *S. hortensis* (Table 2). The variance analysis performed on the DPPH scavenging activity of the essential oils showed significant differences among species ($p \leq 0.05$) and that the essential oil of *M. spicata* was the most potent of all the oils. Therefore, some compounds such as compound Carvone, 1,8-Cineole and Borneol were responsible for the DPPH scavenging effects of *M. spicata* (Mahdavikia and Saharkhiz, 2015). The essential oil of *Th. vulgaris* possessed slightly higher DPPH scavenging effects than that of *B. persicum* and *S. hortensis.* This meant that the common compound Thymol and high value of Carvacrol played a leading role (Ghasemi-Pirbalouti and Dadfar, 2013).

*Bacterial numbers*

Essential oils treatment, particularly 200, 400 µl $l^{-1}$ *M. spicata* and 200, 400 µl.$l^{-1}$ *Th. vulgaris*, had negative effect on bacterial numbers. Preventing bacterial division in vase water can reduce the occurrence of stem bending (Solgi *et al*., 2009), suggesting that bacteria are a main cause. A positive correlation between the number of bacteria and water uptake of the flower stem have been reported (Van Doorn *et al*., 1989). Vascular occlusions in cut rose flower usually develop when the number of bacteria in vase water reach 7-11 $Log_{10}$ CFU/ml (Van Doorn *et al*., 1990). Some antimicrobial compounds have been used in tests with cut flowers. These compounds included silver nitrate (Nair *et al*., 2003), nano-Silver (Nazemi Rafi and Ramezanian, 2013), 8-hydrox-

Table 2 - Antioxidant activity of essential oil extracts of *B. persicum*, *M. spicata*, *Th. vulgaris* and *S. hortensis*

| DPPH | *B. persicum* | *M. spicata* | *Th. vulgaris* | *S. hortensis* |
|---|---|---|---|---|
| $IC_{50}$ (mg/mL) | 183.5 ± 2.15 b | 54.19 ± 0.87 d | 153.52 ± 2.66 c | 258.16 ± 1.53 a |

Mean separation by Tukey Test, P≤0.05.

yquinoline citrate (Solgi *et al*., 2009), chlorine bleach, dichloroisocyanuric acid (Jones and Hill, 1993) and essential oils such as carvacrol (Nazemi Rafi and Ramezanian, 2013) and thymol (Solgi *et al*., 2009). The inhibitory effect of some essential oils on bacterial growth has been attributed to alcohols, esters, aldehydes, phenols, carvacrol, thymol, and eugenol (Bassole and Juliani, 2012). Burt (2004) reported that essential oil components including thymol and carvacrol partition in the lipids of the cell membrane, rendering the membrane permeable and leading to leakage of cell contents, thus exerting their antibacterial action.

*Relative fresh weight and total water uptake*

As expected, relative fresh weight continuously increased early days of experiment (Table 3). Nevertheless, such increases were significantly prolonged by using essential oils in hold solution. The relative fresh weight in 200 µl.l$^{-1}$ *M. spicata* essential oils treated cut flowers was initially about 105.2% (day 1), and increased to 130.1% over 6 days of holding, but in untreated (control) cut flowers, relative fresh weight was initially about 111.3% (day 1), and decreased over 4 days of holding to 105%. Similarly, as with different concentrations of *M. spicata* essential oil, a positive effect of other essential oils on relative fresh weight was confirmed by their impact on duration of increase and percentage of fresh weight. Total water uptake of cut flowers under various treatments from day 1 to 8 in control as well as in all treated samples (Table 3). The maximum uptake of vase solution was found 1.14-fold increase in the treated flower with 200 µl.l$^{-1}$ *Th. vulgaris* as compared to untreated (control) cut flowers. All treated flowers always took up water more than the control.

Water status is a factor directly correlated with the vase life of rose cut flower. This factor is determined by the balance between water uptake and loss due to transpiration (Fanourakis *et al*., 2016). Water uptake depends on variation of cultivars (Fanourakis *et al*., 2016), the viscosity of the vase solution, vascular conductivity and the osmosis gradient between vase solution and stem solute (Alaey *et al*., 2011). The improved water uptake in this study may be due to possible antibacterial activity of essential oils by inhibiting vascular blockage and/or increasing osmosis gradient. The water lose was affected by treatments and essential oils increased water retaining capacity compared to control treatment (without essential oil). It may be due to positive regulatory role of essential oils on stomatal closure which regulates the rates of transpiration and decreases the water loss of leaves and petals (Solgi *et al*., 2009).

*Flower opening*

Generally, the opening of cut flowers kept in essential oils vase solutions was more than those flowers kept in distilled water (control). Also maximum opening of control flowers occurred earlier. Time and diameters of maximum opening flower were different between treatments. However, maxi-

Table 3 - Vase life, total solution uptake, electrolyte leakage, petal anthocyanin content, soluble sugars of petal and bacterial numbers of cut rose (*Rosa hybrida* L.) cv. Bacara treated with *B. persicum*, *M. spicata*, *Th. vulgaris* and *S. hortensis*

| Characteristic | Vase life (days) | Total solution uptake (g) | Electrolyte leakage (%) | Petal anthocyanins content (mg g$^{-1}$ FW) | Soluble sugars of petal (mg g$^{-1}$ FW) | Ascorbate peroxidase activity (Units min$^{-1}$ mg$^{-1}$ protein) | Bacterial numbers (Log10 CFU/ml) | Relative FW in 10$^{th}$ day (%) |
|---|---|---|---|---|---|---|---|---|
| Control | 6.70 e | 98.350 f | 60.6 a | 5.24 d | 1.41 f | 22.6 h | 8.2 a | 79.41 g |
| *B. persicum* 100 µl.l$^{-1}$ | 8.70 bc | 103.25 e | 51.4 b | 6.52 bc | 1.70 f | 25.1 g | 8.1 a | 94.72 cd |
| *B. persicum* 200 µl.l$^{-1}$ | 9.80 b | 106.44 b | 48.5 bc | 7.37 ab | 2.29 e | 24.6 gh | 7.8 ab | 100.80 ab |
| *B. persicum* 400 µl.l$^{-1}$ | 7.90 cd | 100.23 e | 45.7 c | 6.07 c | 2.14 e | 22.9 h | 7.3 d | 84.22 f |
| *M. spicata* 100 µl.l$^{-1}$ | 11.2 a | 110.31 a | 19.8 i | 7.95 a | 4.52 b | 54.9 a | 6.1 f | 104.50 a |
| *M. spicata* 200 µl.l$^{-1}$ | 10.4 ab | 108.65 ab | 28.3 g | 7.68 a | 4.05 bc | 46.7 b | 5.7 g | 95.00 c |
| *M. spicata* 400 µl.l$^{-1}$ | 8.70 bc | 106.71 b | 33.1 f | 6.03 c | 3.77 c | 43.3 bc | 5.5 gh | 91.48 de |
| *Th. Vulgaris* 100 µl.l$^{-1}$ | 8.30 c | 105.94 c | 22.4 h | 7.19 b | 4.35 b | 38.6 d | 7.5 cd | 91.71 de |
| *Th. Vulgaris* 200 µl.l$^{-1}$ | 10.6 ab | 112.67 a | 17.4 j | 7.70 a | 5.18 a | 40.8 cd | 5.5 gh | 104.50 a |
| *Th. Vulgaris* 400 µl.l$^{-1}$ | 9.20 b | 107.36 b | 32.0 f | 7.36 ab | 3.95 c | 41.0 c | 5.3 h | 98.54 bc |
| *S. hortensis* 100 µl.l$^{-1}$ | 7.80 d | 98.670 f | 41.5 d | 5.83 c | 2.35 e | 33.7 ef | 7.8 bc | 83.54 fg |
| *S. hortensis* 200 µl.l$^{-1}$ | 8.60 bc | 103.68 de | 38.5 e | 6.44 bc | 3.28 d | 32.1 f | 7.2 d | 87.83 ef |
| *S. hortensis* 400 µl.l$^{-1}$ | 9.10 bc | 104.86 d | 37.5 e | 6.87 b | 3.46 cd | 35.9 e | 6.8 e | 97.87 bc |

Mean separation for each parameter within rows by Tukey Test, P≤0.05.

mum diameter was observed with flowers kept in 100 µl.l$^{-1}$ *M. spicata* essential oil vase solution (Fig. 1). Mechanisms of flower opening vary for different flowers and are sensitive to various environmental conditions such as temperature, light, carbohydrate supply (Kumar *et al*., 2008) and water relations. During the opening, numerous proceedings take place in a well-defined sequence, representing all parts of plant development, such as division, differentiation and elongation of cell (Kumar *et al*., 2008) or gene expression (Hoeberichts *et al*., 2005). In an experiment, essential oils at some concentrations had a noticeable effect on promoting flower opening of cut rose flowers. However, the treatments with a relatively higher concentration of essential oils showed a less effect on flower opening. According to Mahdavikia and Saharkhiz (2015) the function of essential oils as an effective antioxidant mainly depends on its concentration. It has been demonstrated that a relatively high dose of essential oils may be associated with injury of membranes and nucleic acids and cell death (Ghasemi-Pirbalouti and Dadfar, 2013). The results suggest that essential oils at effective concentrations maintained water uptake and inhibited vascular occlusions development. Thus, essential oils may improve water relations and supplementation of carbon sources, leading to cell expansion and flower opening.

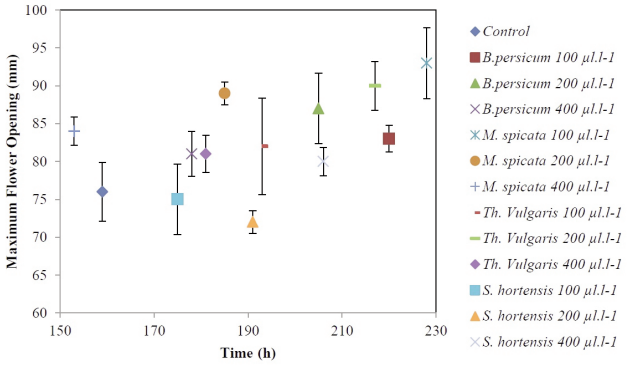



Fig. 1 - Time of maximum flower opening. Values are given as mean±SD of three replicates.

*Petal anthocyanin content*

Petal anthocyanin content in cut flowers was determined. Cut flowers treated with 100 µl.l$^{-1}$ *M. spicata* essential oil in hold solution were observed to accumulate the highest rates of anthocyanin in the petals. Other treatments led to higher amounts of anthocyanins compared to untreated (control) cut flowers. Generally, all cut flowers treated with essential oils showed a similar tendency to maintain anthocyanin Petal, however, essential oils of *Th. vulgaris* and *M. spicata* were more efficient than *B. persicum* and *S. hortensis*.

One of plant defense system is the presence of endogenous antioxidative compounds such as anthocyanins (Apel and Hirt, 2004). Singlet oxygen produced can be detoxified by anthocyanins and affect the vase life. Accumulation of these pigments in cell vacuoles, their hue and intensity depend on external conditions. Anthocyanin biosynthesis is an essential part of flower development (Dela *et al*., 2003). In the primary developmental phases of the flower, the anther produces a signal or GA, which promotes petal pigmentation (Kumar *et al*., 2008). So, in this study, flower opening and development could be effect on anthocyanins accumulation. Additionally, earlier studies have indicated that sugars such as sucrose are required as substrates for anthocyanin biosynthesis (Nagira and Ozeki, 2004). Here, we showed that essential oils increased petal soluble sugar content. It seems that there is an interaction between the soluble sugars concentration and anthocyanins.

*Ascorbate peroxidase activity*

With cut roses, the highest ascorbate peroxidase activity in petals occurred in those treated with *M. spicata* essential oils at the concentration of 100 µl l$^{-1}$. As shown in Table 3, the activity of ascorbate peroxidase in control flowers was less than that of the flowers treated with essential oils, however, there were no significant difference between control flowers and flowers treated with different concentrations of *B. persicum* essential oils. Ascorbate peroxidase enzyme is considered to play significant roles in cellular defense against stresses (Sevillano *et al*., 2009). During the later stages of vase life, petals contain higher levels of reactive oxygen species leading to oxidative damage (Hossain *et al*., 2006). Generally, treatment with essential oils increased ascorbate peroxidase activity compared the control. Higher levels of the enzymatic activity in treated flowers were likely to counteract the oxidative stress and to scavenge the active oxygen species alleviate to the oxidative stress induced in cut roses and thus delayed flower senescence. These results are in agreement with the findings of Hasan *et al*., (2014) who mentioned that cut roses were of an efficient antioxidant defense system.

*Electrolyte leakage*

The ability to increase membrane stability is a characteristic of drought tolerance strategy (Turner, 1986). With control flowers, a significant increase in

electrolyte leakage was detected; however, treatment with any essential oils level significantly inhibited electrolyte leakage increase relative to the control (Table 3). The treatment with 200 µl $l^{-1}$ *Th. vulgaris* essential oils resulted in the lowest electrolyte leakage followed by 100 µl $l^{-1}$ *M. spicata* essential oils. Wilting in cut flower is often accompanied by membrane damage resulting in the leakage of solutes (Ye *et al*., 2000). With studied cut flower, such as day lily (Panavas *et al*., 1998), the most changes in the rate of electrolyte leakage were observed during the late stage of vase life. Maintenance of membrane stability in response to essential oils application was most likely due to induced reduction of lipid peroxidation. This is supported by a lower level of electrolyte leakage in essential oil treatments.

*Soluble sugars*

Another factor controlling vase life of cut rose flower is sugar content, as the carbon supply is cut (Halevy and Mayak, 1979). The essential oils had a strong influence on the soluble sugars concentration in the petal of cut flowers and all treatments led to significantly more soluble sugars. Soluble sugars increased approximately 3.6 fold from 1.41 mg $g^{-1}$ FW for the control to 5.18 mg $g^{-1}$ FW for pulsing flowers in solutions containing essential oil of *Th. vulgaris* (200 µl $l^{-1}$) and to 4.52 mg $g^{-1}$ FW using 100 µl $l^{-1}$ *M. spicata* essential oil. With cut flowers treated with preservative solution, minimum soluble sugar contents were observed in flowers kept in solutions containing 100 µl $l^{-1}$ *B. persicum* essential oils. It seems that essential oils can change the capacity for sugar uptake in petals and stimulate active sucrose uptake. Translocation of soluble sugars is considered as a key factor affecting the vase life (Khayat and Zieslin, 1989). The addition of chemicals including some componds such as silver nitrate, aluminum sulphate and 8-hydroxyquinoline sulphate to holding solutions has been tried with varied success in efforts to control vascular blockage and prolong the vase life of cut roses (Van Doorn *et al*., 1990). However, there is no available evidence on the use of essential oils for control of microbial contaminations and holding soluble sugars petal of cut flowers such as rose.

*Vase life*

Cut flower wilting is a widely reported problem during vase life of rose, particularly when xylem vessels are blocked by microorganisms (Damunupola and Joyce, 2008). In this study, longevity of treated cut flowers was significantly improved compared with the untreated (control) cut flower. Also, significant differences were found in longevity conferred by the essential oils of species and concentrations. However, *B. persicum, M. spicata* and *Th. vulgaris* decreased vase life at concentrations more than 200 µl $l^{-1}$, 100 µl $l^{-1}$ and 200 µl $l^{-1}$, respectively. The treatment with *M. spicata* 200 µl $l^{-1}$ essential oil almost doubled the vase life of cut roses. Proper doses of some essential oils at proper dose could delay senescence in selected fruits (Ramezanian *et al*., 2016) and cut flowers (Solgi *et al*., 2009). For example, Solgi *et al*. (2009) concluded that the vase life of gerbera flowers was extended by the 50 or 100 mg $l^{-1}$ carvacrol from 8.3 to 16 days. In the present study, similar results was obtained with the vase life of cut rose.

## 4. Conclusions

The naturally occurring compounds in essential oils, such as thymol, carvone, carvacrol and menthol showed different levels of antibacterial activity. The major qualitative trait of cut roses is their vase life, which mainly depends on the water uptake of the stems after harvest and is hampered by the presence of bacteria. Also, at relatively high concentration of the essential oils tested, early flower opening and senescence were related to an effect other than bacteria. This effect was correlated with low relative fresh weight and water uptake. It cannot be excluded at present that this early senescence might be due to a toxic effect. More information regarding the action of essential oils during vase life is required for better understanding of the mechanism of petal senescence of rose.

## Acknowledgements

This work was granted by Khuzestan Ramin University of Agriculture and Natural Resources.

## References

ALAEY M., BABALAR M., NADERI R., KAFI M., 2011 - *Effect of pre - and postharvest salicylic acid treatment on physio-chemical attributes in relation to vase-life of rose cut flowers.* - Postharvest Biol. Technol., 61: 91-94.

APEL K., HIRT H., 2004 - *Reactive oxygen species: metabolism, oxidative stress and signal transduction.* - Ann. Rev. Plant Biol., 55: 373-399.

BALESTRA G.M., AGOSTINI R., BELLINCONTRO A., MENCARELLI F., VARVARO L., 2005 - *Bacterial populations related to gerbera (*Gerbera jamesonii *L.) stem break.* -

Phytopathol. Mediterr., 44: 291-299.

BASSOLE I.H.N., JULIANI H., 2012 - *Essential oils in combination and their antimicrobial properties.* - Molecules, 17: 3989-4006.

BEN EL HADJ ALI I., BAHRI R., CHAOUACHI M., BOUSSAID M., HARZALLAH-SKHIRI F., 2014 - *Phenolic content: antioxidant and allelopathic activities of various extracts of* Thymus numidicus *Poir.- organs.* - Ind. Crops. Prod., 62: 188-195.

BOTELHO M.A., NOGUEIRA N.A.P., BASTOS G.M., FONSECA S.G.C., LEMOS T.L.G., MATOS F.J.A., MONTENEGRO D., HEUKELBACH J., RAO V.S., BRITO G.A.C., 2007 - *Antimicrobial activity of the essential oil from* Lippia sidoides*, carvacrol and thymol against oral pathogens.* - Braz. J. Med. Biol. Res., 40: 349-356.

BRAND-WILLIAMS W., CUVELIER M.E., BERSET C., 1995 - *Use of a free radical method toevaluate antioxidant activity.* - LWT-Food Sci. Technol., 28: 25-30.

BURT S., 2004 - *Essential oils: their antibacterial properties and potential applications in foods - A review.* - Int. J. Food Microbiol., 94: 223-253.

CAIRNS T., YOUNG M., ADAMS J., EDBERG B., 2000 - *Modern roses XI: The world encyclopedia of roses.* - Academic Press, Cambridge, MA, USA, pp. 11-12.

CHAUHAN R.S., KAUL M.K., SHAHI A.K., KUMAR A., RAM G., TAWA A., 2009 - *Chemical composition of essential oils in* Mentha spicata *L. accessions from Northwest Himalayan region, India*. - Ind. Crops Prod., 29: 654-656.

DAMUNUPOLA J.W., JOYCE D.C., 2008 - *When is a vase solution biocide not, or not only, antimicrobial?* - Jpn. Soc. Hortic. Sci., 77: 211-228.

DELA G., OR E., OVADIA R., NISSIM-LEVI A., WEISS D., OREN-SHAMIR M., 2003 - *Changes in anthocyanin concentration and composition in 'Jaguar' rose flowers due to transient high air temperature conditions*. - Plant Sci., 164: 333-340.

DE WITTE Y., VAN DOORN W.G., 1988 - *Identification of bacteria in the vase water of roses, and the effect of the isolated strains on water uptake.* - Sci. Hort., 35: 285-291.

FANOURAKIS D., GIDAY H., LI T., KAMBOURAKIS E., LIGOXIGAKIS E.K., PAPADIMITRIOU M., STRATARIDAKI A., BOURANIS D., HEUVELINK E., OTTOSEN C., 2016 - *Antitranspirant compounds alleviate the mild-desiccation-induced reduction of vase life in cut roses*. - Postharvest Biol. Technol., 117: 110-117.

FROHLICH M., KUTSCHERAH U., 1995 - *Changes in soluble sugars and proteins during. Development of rye coleoptiles.* - J. Plant Physiol., 146: 121-125.

GHASEMI-PIRBALOUTI A., DADFAR S., 2013 - *Chemical constituents and antibacterial activity of essential oil of* Satureja bachtiarica *(Lamiaceae).* - Acta Pol. Pharm., 70: 933-938.

GHASEMI-PIRBALOUTI A., VOSOGHI N., CRAKER L., SHIRMARDI H.A., 2014 - *Chemical composition of the essential oil of Satureja kallarica Jamzad.* - J. Essent. Oil Res., 26: 228-231.

HALEVY A.H., MAYAK S., 1979 - *Senescence and postharvest physiology of cut flowers, part 1.* - Hortic. Rev., 1: 204-236.

HASAN F.A.S., ALI E.F., EL-DEEB B., 2014 - *Improvement of post harvest quality of cut rose cv. 'First Red' by biologically synthesized silver nanoparticles*. - Sci. Hortic., 179: 340-348.

HE S.G., JOYCE D.C., IRVING D.E., FARAGHER J.D., 2006 - *Stem-end blockage in cut* Grevillea *'Crimson Yul-lo' inflorescences.* - Postharvest Biol. Technol., 41: 78-84.

HOEBERICHTS F.A., DE JONG A.J., WOLTERING E.J., 2005 - *Apoptotic like cell death marks the early stages of gypsophilla (*Gypsophila paniculata*) petal senescence*. - Postharvest Biol. Technol., 35: 229-236.

HOSSAIN Z., MANDAL A.K.A., DATTA S.K., BISWAS A.K., 2006 - *Decline in ascorbate peroxidase activity-a prerequisite factor for tepal senescence in gladiolus.* - J. Plant Physiol., 163: 186-194.

JONES R.B., HILL M., 1993 - *The effect of germicides on the longevity of cut flowers*. - J. Amer. Soc. Hortic. Sci., 118: 350-354.

KAR M., MISHRA D., 1976 - *Catalase, peroxidase and polyphenoloxidase activities during rice leaf senescence.* - Plant Physiol., 57: 315-319.

KHAYAT E., ZIESLIN N., 1989 - *Translocation of $^{14}C$ carbohydrate content and activity of the enzymes of sucrose metabolism in rose petals at different night temperatures.*- Physiol. Plant., 76: 581-585.

KUMAR N., SRIVASTAVA G.C., DIXIT K., 2008 - *Flower bud opening and senescence in roses (*Rosa hybrida *L.).* - Plant Growth Regul., 55: 81-99.

LUTTS S., KINET J.M., BOUHARMONT J., 1996 - *NaCl-induced senescence in leaves of rice (*Oryza sativa *L.) cultivars differing in salinity resistance.* - Annals. Bot., 78: 389-398.

MAHDAVIKIA F., SAHARKHIZ M.J., 2015 - *Phytotoxic activity of essential oil and water extract of peppermint (*Mentha *×* piperita *L. cv. Mitcham).* - J. Appl. Res. Med. Aromatic Plants, 2: 146-153.

NAGIRA Y., OZEKI Y., 2004 - *A system in which anthocyanin synthesis is induced in regenerated torenia shoots.* - J. Plant Res., 117: 377-383.

NAIR S.A., SINGH V., SHARMA T.V.R.S., 2003 - *Effect of chemical preservatives on enhancing vase-life of gerbera flowers*. - J. Trop. Agric., 41: 56-58.

NAZEMI RAFI Z., RAMEZANIAN A., 2013 - *Vase life of cut rose cultivars 'Avalanche' and 'Fiesta' as affected by nano-silver and S-carvone treatments.* - S. African J. Bot., 86: 68-72.

PANAVAS T., WALKER E.L., RUBINSTEIN B., 1998 - *Possible involvement of abscisic acid in senescence of daylily petals.* - J. Exp. Bot., 49: 1987-1997.

RAMEZANIAN A., AZADI M., MOSTOWFIZADEH-GHALAMFARSA R., SAHARKHIZ M.J., 2016 - *Effect of* Zataria mul-

tiflora *Boiss and* Thymus vulgaris *L. essential oils on black rot of 'Washington Navel' orange fruit.* - Postharvest Biol. Technol., 112: 152-158.

SCHERER R., LEMOS M.F., LEMOS M.F., MARTINELLI G.C., MARTINS J.D.L., DA SILVA A.J., 2013 - *Antioxidant and antibacterial activities and composition of Brazilian spearmint (*Mentha spicata *L.).* - Ind. Crops Prod., 50: 408-413.

SEVILLANO L., SANCHEZ-BALLESTA M.T., ROMOJARO F., FLORES F.B., 2009 - *Physiological, hormonal and molecular mechanisms regulating chilling injury in horticultural species. Postharvest technologies applied to reduce its impact.* - J. Sci. Food Agric., 89: 555-573.

SHAHSAVARI N., BARZEGAR M., SAHARI M.A., NAGHDIBADI H., 2008 - *Antioxidant activity and chemical characterization of essential oil of* Bunium persicum*.* - Plant Foods Hum. Nutr., 63: 183-188.

SHARIFIFAR F., MOSHAFI M.H., MANSOURI S.H., KHODASHENAS M., KHOSHNOODI M., 2007 - In vitro *evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic* Zataria multiflora *Boiss.* - Food Control., 18: 800-805.

SOLGI M., KAFI M., TAGHAVI T.S., NADERI R., 2009 - *Essential oils and silver nanoparticles (SNP) as novel agents to extend vase-life of gerbera (*Gerbera jamesonii *cv. 'Dune') flowers.* - Postharvest Biol. Technol., 53: 155-158.

TURNER N.C., 1986 - *Crop water deficit: a decade of progress*. - Adv. Agron., 39: 1-51.

VAN DER SMAN R.G.M., EVELO R.G., WILKINSON E.C., VAN DOORN W.G., 1996 - *Quality loss in packed rose flowers due to* Botrytis cinerea *infection as related to temperature regimes and packaging design.* - Postharvest Biol. Technol., 7: 341-350.

VAN DOORN W.G., DE WITTE Y., PERIK R.J., 1990 - *Effect of antimicrobial compounds on the number of bacteria in stems of cut rose flowers.* - J. Appl. Bact., 68: 117-122.

VAN DOORN W.G., SCHURER K., DE WITTE Y., 1989 - *Role of endogenous bacteria in vascular blockage of cut rose flowers.* - J. Plant Physiol., 134: 375-381.

WAGACHA J.M., MUTHOMI J.W., 2008 - *Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies.* - Int. J. Food Microbiol., 124: 1-12.

YAHYAZADEH M., OMID-BAIGI R., ZARE R., TAHERI H., 2008 - *Effect of some essential oils onmycelial growth of* Penicillium digitatum *Sacc.* - World J. Microbiol. Biotechnol., 24: 1445-2145.

YE Z., RODRIGUEZ R., TRAN A., HOANG H., DE LOS SANTOS D., BROWN S., VELLANOWETH L., 2000 - *The developmental transition to flowering repress ascorbate peroxidase activity and induces enzymatic lipid peroxidation in leaf tissue in* Arabiodopsis thaliana*.* - Plant Sci., 58: 115-127.

AHS
Advances in Horticultural Science

# Phytochemical composition and insecticidal potentials of some plant aqueous extracts in suppressing *Podagrica* spp. (Coleoptera: Chrysomelidae) infestation on Okra (*Abelmoschus esculentus* L. Moench)

**J.M. Adesina[1, 2 (*)], Y. Rajashekar [2]**

[1] *Department of Crop, Soil and Pest Management Technology, Rufus Giwa Polytechnic, P.M.B. 1019, Owo, Ondo State, Nigeria.*

[2] *Insect Chemical Ecology Laboratory, Institute of Bioresources and Sustainable Development, Department of Biotechnology, Government of India, Takyelpat, Imphal 795001, Manipur, India.*

OPEN ACCESS

*Key words*: *Bryscocarpus coccineus*, *Calotrophus procera, Canarium schweinfurthii*, insecticidal activity, phytochemical screening.

**(*) Corresponding author:**
moboladesina@yahoo.com

**Citation:**
ADESINA J.M., RAJASHEKAR Y., 2018 - *Phytochemical composition and insecticidal potentials of some plant aqueous extracts in suppressing Podagrica spp. (Coleoptera: Chysomelidae) infestation on Okra (Abelmoschus esculentus L. Moench)* - Adv. Hort. Sci., 32(1): 71-78



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 8 September 2017
Accepted for publication 12 January 2018

**Abstract: Foliar application of 25% w/v crude aqueous extracts of *Calotrophus procera* (Aiton) W.T. Aiton, *Canarium schweinfurthii* (Engl.) and *Bryscocarpus coccineus* (Schum. & Thonn.) were evaluated for their insecticidal activity in reducing *Podagrica* infestation on okra. Results showed that the plants extracts significantly suppress *Podagrica* spp. infestation and protect okra plant from severe leaves defoliation, with *C. schweinfurthii* (21.67 and 20.14) and *B. coccineus* (23.07 and 24.55) showing promising insecticidal activity for both cropping seasons. The yield attributes from okra sprayed with Lambda cyhalothrin did not differ significantly compared to those sprayed with botanical insecticide despite having highest yield attributes. Qualitative phytochemical screening revealed the presence of Triterpenoids, Steroids, Flavonoids, Phlobatanins, Saponins, Tannins, Cardiac glycoside and Anthraquinones. Alkaloids and Anthraquinones were not detected in *C. procera* and *C. schweinfurthii* while Triterpenoids and Phlobatanins were absent in *C. schweinfurthii.* The presence of these phytochemicals indicates that the plants possess insecticidal properties responsible for significant reduction in *Podagrica* spp. infestation, severity of leaves damaged and improved okra yields. Performance of the treatments is rated in the following order: Lambda cyhalothrin > *B. coccineus* > *C. schweinfurthii* > *C. procera,* with *B. coccineus* and *C. schweinfurthii* having similar treatment means in all the parameters evaluated. In light of the foregoing, crude extracts of *B. coccineus* and *C. schweinfurthii* could be utilized as suitable alternative to synthetic insecticide in sustainable okra production.**

## 1. Introduction

Okra (*Abelmoschus esculentus* L. Moench) is an important fruit veg-

etable crop in the diets of most people in the tropics and subtropical countries. It is grown mainly for its freshly immature pods and ranks fourth worldwide after pepper, tomato and onion on the basis of land area, production and value.

Despite the great demand for okra due to its nutritional and economic importance, okra production is being hampered by array of insect pest infestation resulting in poor yield and low market value. Different growth stages of okra are generally attacked by different insect pests (Adesina and Afolabi, 2014; Kedar *et al*., 2014) and *Podagrica* species (Coleoptera: Chrysomelidae) is one of the most prevalent and damaging insect pests considered as major constraint to cultivation of okra which defoliate or damage the plant leaves and flowers; thereby results in reduction of the photosynthetic capability of the crop (Odebiyi, 1980). The insects equally act as vectors in transmitting mosaic viral diseases and ultimately reduce yield (Fasunwon and Banjo, 2010), mainly if control measures are not undertaken. Important yield losses of 20-50% are reported in Nigerian and Ghana (Obeng-Ofori and Sackey, 2003; Ahmed *et al.*, 2007; Fajinmi and Fajinmi, 2010).

Currently, synthetic insecticide is being used across the globe to control agriculturally important insect pests due to their quick action and lasting effect (Alao *et al.*, 2011). The failure of synthetic insecticides to ensure total insect pest control due to development of resistant and resurgence and couple with increasing concern over environmental pollution, carcinogenic effect and destruction of beneficiary insects (Isman, 2008), level of pesticide residues in food had compelled the scientific community to explore the abandoned and neglected traditional and indigenous products that are cheap, environmental safe and easily biodegradable products as alternatives to synthetic insecticides for tackling agricultural insect pests (Antonio, 2009; Alao *et al*., 2011; Aetiba and Osekre, 2016).

Presently, in many parts of the world, attention has been focused on the utilization of plant products that possess both medicinal and aromatic properties which are abound in various agro-ecological zones of the world as novel chemotherapeutants in plant protection (Dubey *et al*., 2010). The popularity of botanical pesticides is once again increasing and some plant products either in crude form or by processing into different formulations are being used globally as green pesticides (Dubey *et al*., 2008).

The shortcomings associated with the use of synthetic chemicals, necessitated the idea of developing effective, cheap and easily biodegradable alternative products. Therefore, this study aims to screen and report the efficacy of aqueous extracts of *Calotrophus procera* (Ait.) Ait*., Canarium schweinfurthii* (Engl.) and *Bryscocarpus coccineus* (Shum and Thonn.) in suppressing flea beetles *Podagrica* spp. (Coleoptera: Chrysomelidae) infestation on okra.

## 2. Materials and Methods

*Experimental location, designs and treatments*

The study was carried out on 168 $m^2$ area of land manually cleared and prepared at the Teaching and Research Farm, Rufus Giwa Polytechnic (RUGIPO), Owo, Ondo State located in South Western Nigeria and lies on latitude 7° 12’ N and longitude 7° 35’ E. The experiment was laid out in a Randomized Complete Block Design (RCBD) with three replications. The experimental land was divided into 4 blocks of 4x4 m to produce a total of 12 plots. The plot size was 2x1.5 m with 0.5 m between plots and 1x1 m between blocks to prevent pesticide drift and inter-plot interference, respectively.

An early maturity okra cultivar (NH-47) obtained from Ondo state Agricultural Development Project (ADP), Akure, Ondo State, Nigeria was sown with two to three seeds per hole at a depth of 2-3 cm and spaced 50x60 cm and later thinned to one plant per stand. Weeding was carried out manually as at when due to ensure clean plots free from weed competition.

The treatments applied were 25% w/v *B. coccineus, C. schweinfurthii, C. procera* and 0.8 L/ha synthetic insecticide (Lambda cyhalothrin) as control. Application of treatments commenced four weeks after planting (WAP) with a 2-litre capacity hand-held sprayer. The spraying was carried out weekly till fruiting early in the morning to avoid photo decomposition and drifting of the extracts. Synthetic insecticide was applied every two weeks.

*Collection and preparation of plant extracts*

Leaves of *B. coccineus* and *C. schweinfurthii* were collected from Owo, Ondo state, Nigeria and *C. procera* were collected from Ogbagi Akoko Ondo State, Nigeria (7° 35’ N, 5° 43’ E) at full blooming and 25 kg were weighed separately using electronic balance (Table 1). Thereafter, the weighed plants were washed with water to remove dirt or other contaminants and each of the plant materials was pounded separately in a mortar into a fine soft paste form. The

Table 1 - Plant materials used for the experiment

| Scientific name | Common name | Family | Part used |
|---|---|---|---|
| *Calotropis procera* | Giant swallow wort or Milkweed | Asclepiadaceae | leaves |
| *Canarium schweinfurthii* | African elemi or canarium | Burseraceae | leaves |
| *Byrsocarpus coccineus* | Short-pod | Connaraceae | leaves |

paste (crushed plant leaves) was then put into a 10-litre plastic bucket and the appropriate volume of distilled water added to make a 25% w/v crude aqueous extract solution (25% w/v represents an extract made with 25 g of crushed plant leaves per every 100 ml of water). The pastes were soaked overnight (approximately 14 hours) in a covered bucket with occasional stirring and filtered using muslin cloth with the filtrates stored in a 10 L keg in a cool dry place till use.

*Phytochemical screening*

The qualitative phytochemical tests were performed on the crude aqueous extract to detect the presence of bioactive secondary metabolites in tested plant materials using standard laboratory methods following the procedure described by Odebiyi and Sofowora, 1978; Sofowora, 1982; Williamson *et al.,* 1996; Banso and Ngbede, 2006; Ngbede *et al*., 2008.

*Data collection and statistical analysis*

All data were collected from five plants randomly tagged per treatment in the two middle rows of each plot. Leaves damage index was determined by visual counting of the number of holes caused by the insect feeding activities. While insect count for estimation of the population densities of *P. uniformis* and *P. sjostedti* was made 4 WAP between the hours 6-7 AM when the insects are still inactive through visual counting. The fruits were harvested two months after planting (MAP), when the fruits were still fresh at the interval of 4 days. Weighing balance was used to determine the weight of the fruits and the fruits length measured using ruler. Data collected were analyzed by analysis of variance (ANOVA) using Gester Version 1.2, insect count were subjected to square root (y =√) transformation before analysis to normalize the data. Significant treatment means were compared using Tukey test at 5% probability.

## 3. Results

*Podagrica spp. population before application of some plant aqueous extracts*

Table 2 shows *Podagrica spp.* population on okra before the application of the aqueous plant extracts treatments. The results showed that the insect populations were not significantly different. The population ranges between 4.07-4.93/plant for 2015 cropping season and 4.10-4.98/plant for 2016 cropping season.

Table 2 - *Podagrica* spp. population before application of some plant aqueous extracts

| Treatments | Insect population | |
|---|---|---|
| | 2015 | 2016 |
| *C. procera* | 4.07±0.77 a | 4.98±0.15 a |
| *C. schweinfurthii* | 4.08±0.66 a | 4.46±0.34 a |
| *B. coccineus* | 4.27±0.38 a | 4.10±0.07 a |
| Lambda cyhalothrin | 4.93±0.66 a | 4.47±0.28 a |

Treatments with the same letter in column are not statistically significant different from each other.

*Toxicity effect of some plant aqueous extracts in suppressing Podagrica spp. infestation*

The utilization of plant aqueous extracts to suppress *Podagrica* spp. infestation on okra was presented in Tables 3 and 4 for 2015 and 2016 cropping season, respectively. Application of the various treat-

Table 3 - Toxicity effect some plant aqueous extracts in suppressing *Podagrica* spp. infestation 2015

| Treatments | 4 WAP | | 5 WAP | | 6 WAP | | 7 WAP | | 8 WAP | | 9 WAP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS |
| *C. procera* | 2.93±1.86 a | 0.33±0.31 ab | 0.80±0.60 ab | 0.87±1.17 a | 1.80±1.51 a | 3.27±1.36 b | 1.33±1.03 a | 0.67±0.12 a | 0.20±0.20 b | 0.60±0.35 a | 0.33±0.31 a | 0.80±0.31 ab |
| *C. schweinfurthii* | 1.13±1.14 ab | 0.07±0.12 c | 0.27±0.23 c | 0.07±0.12 b | 0.37±0.35 b | 1.13±0.70 a | 0.27±0.35 ab | 0.20±0.35 a | 0.00 | 0.53±0.61 a | 0.18±0.12 a | 0.73±0.51 ab |
| *B. coccineus* | 1.40±1.22 ab | 0.6±0.87 a | 1.0±1.40 a | 0.40±0.53 a | 0.22±0.06 b | 0.47±0.53 a | 0.4±0.69 ab | 0.80±1.22 a | 0.82±1.05 a | 0.6±0.53 a | 0.8±1.22 a | 0.93±0.52 a |
| Lambda cyhalothrin | 0.13±0.23 c | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00a | 0.00 |

WAP = Weeks after planting; DAS = Days after spray.
Treatments with the same letters in columns are not statistically significant different from each other.

Table 4 - Toxicity effect some plant aqueous extracts in suppressing *Podagrica* spp. infestation 2016

| Treatments | 4 WAP | | 5 WAP | | 6 WAP | | 7WAP | | 8 WAP | | 9 WAP | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS | 3 DAS | 7 DAS |
| *C. procera* | 2.05±0.62 a | 0.73±0.31 a | 0.98±0.56 a | 0.53±1.04 a | 1.18±1.51 a | 1.02±0.16 a | 0.88±1.06 a | 0.63±0.14 b | 0.42±0.20 b | 0.6±0.35 a | 0.55±0.24 a | 0.56±0.31 a |
| *C. schweinfurthii* | 1.89±0.14 ab | 0.28±1.52 b | 0.67±0.23 a | 0.18±0.32 a | 0.37±0.35 ab | 0.63±1.08 a | 0.41±0.05 ab | 0.4±0.33 a | 0.20±0.07 b | 0.53±0.61 a | 0.17±0.16 b | 0.41±1.61 a |
| *B. coccineus* | 1.44±0.21 ab | 0.92±0.87 a | 0.82±1.03 a | 0.46±1.07 a | 0.08±0.11 b | 0.82±0.12 a | 0.2±0.09 ab | 0.87±0.22 a | 1.0±0.35 a | 0.6±0.53 a | 0.46±1.42 a | 0.57±0.25 a |
| Lambda cyhalothrin | 0.10±0.23 c | 0.22±1.61 b | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00a | 0.00 | 12:00 | 0.00 | 0.00 |

WAP = Weeks after Planting; DAS = Days after spray.
Treatments with the same letters in columns are not statistically significant different from each other.

ments resulted in significant reduction in the insect population, while synthetic insecticides completely suppressed the insect pest infestation. The results clearly indicate that insect population reduced in relation to the number of spraying regimes, with the exception of okra plants sprayed with *C. procera* aqueous extract, which slightly increased or resurgence at 6 WAP for both seasons and 3 days after spraying (DAS) at 7 WAP in 2015 cropping season only. In 2015 non-significant difference was observed at 3 DAS and 7 DAS in all the treatments at 7, 8 and 9 WAP, while in 2016 non-significant difference was recorded 7 DAS at 6, 8 and 9 WAP in 2016 cropping season, respectively.

The insecticidal activity of the aqueous plant extracts treatments is rated in the following decreasing order: *B. coccineus* > *C. schweinfurthii* > *C. procera,* with *B. coccineus* and *C. schweinfurthii* having similar treatment means which is not significantly different in most instances but significantly differ compared to *C. procera* in both seasons.

*Toxicity effect of some plant aqueous extracts on okra yield attributes*

The yield of okra plant in response to various plant aqueous extracts evaluated in reducing *Podagrica* spp. infestation was presented in Table 5. From the results, fruit weight and fruit diameter were not significantly different in both cropping seasons for all the treatments sprayed on the okra against *Podagrica* spp. infestation. Also non-significant difference equally observed between okra plants sprayed with *B. coccineus* and *C. schweinfurthii* compared to Lambda cyhalothrin for number of harvested fruit and fruit lengths for both seasons except for *C. schweinfurthii* in 2015 season. Although, there was significant difference in the number of harvested fruits and fruit lengths between okra sprayed with *B. coccineus*, *C. schweinfurthii* and Lambda cyhalothrin compared to *C. procera* in both seasons. The yield attribute performance of the treatments is rated in the following decreasing order: Lambda cyhalothrin > *B. coccineus* > *C. schweinfurthii* > *C. procera,* with *B. coccineus* and *C. schweinfurthii* having similar treatment means.

*Effects of plants aqueous extracts on severity of leaves damage on Okra by Podagrica spp.*

The utilization of plants aqueous extract on severity of leaves damage on Okra (*A. esculentus L.*) by *Podagrica spp.* was presented in Table 6. Significant

Table 5 - Toxicity effect some plant aqueous extracts okra yield attributes

| Parameters | *C. procera* | | *C. schweinfurthii* | | *B. coccineus* | | Lambda cyhalothrin | |
|---|---|---|---|---|---|---|---|---|
| | 2015 | 2016 | 2015 | 2016 | 2015 | 2016 | 2015 | 2016 |
| Number of pod | 3.75±1.13 b | 3.98 ±0.19 b | 4.69±1.59 a | 4.51±1.79 a | 4.75±1.79 a | 4.93±2.41 a | 5.12±0.84 a | 5.45±1.79 a |
| Fruit length | 3.02±0.88 ab | 3.47±1.66 ab | 3.93±2.71 ab | 4.00±1.34 a | 4.12±1.76 a | 4.70±0.28 a | 4.83±2.02 a | 4.67±1.17 a |
| Fruit weight | 0.08±0.06 a | 0.05±0.02 a | 0.05±0.03 a | 0.09±0.11 a | 0.05±0.06 a | 0.07±0.12 a | 0.04±0.03 a | 0.06±0.02 a |
| Diameter | 5.51±1.89 a | 4.80±0.43 a | 4.9±1.45 a | 4.64±0.93 a | 4.44±1.72 a | 4.03±1.06 a | 4.72±0.69 a | 4.46±0.82 a |

Treatments with the same letters in columns are not statistically significant different from each other.

Table 6 - Effects of plants aqueous extracts on severity of leaves damage on Okra by *Podagrica* spp.

| Severity of leaf damage | *C. procera* | | *C. schweinfurthii* | | *B. coccineus* | | Lambda cyhalothrin | |
|---|---|---|---|---|---|---|---|---|
| | 2015 | 2016 | 2015 | 2016 | 2015 | 2016 | 2015 | 2016 |
| 4 WAP | 3.47±1.08 e | 4.51±0.71 e | 4.73±0.43 e | 4.02±0.66 b | 3.40 ±1.83 c | 3.27±1.10 d | 1.67±0.76 a | 1.44 ±0.83 a |
| 5 WAP | 6.27±0.73 e | 8.12±2.46 e | 5.82±1.18 e | 6.83±0.37 b | 4.00±1.95 c | 4.17±1.38 d | 3.20 ±1.51 a | 4.27 ±0.73 a |
| 6 WAP | 12.23±1.09 d | 12. 85±2.03 d | 9.50±1.76 d | 8.91±1.92 b | 10.53±1.68 b | 11.48±2.06 c | 4.07 ±0.69 a | 4.23 ±1.09 a |
| 7 WAP | 19.93±1.93 c | 20.14±1.17 c | 11.97±0.86 c | 13.25±1.81 ab | 16.27 ±2.68 ab | 17.04±1.39 b | 4.07 ±0.39 a | 4.93 ±2.93 a |
| 8 WAP | 29.60±1.55 b | 26.92±2.41 b | 15.53±1.78 b | 17.09±2.01 a | 20.67±2.61 a | 19.85±2.14 ab | 4.80 ±1.70 a | 5.60 ±1.55 a |
| 9 WAP | 32.20±0.66 a | 33.32±1.82 a | 21.67±2.90 a | 20.14±1.26 a | 23.07±1.32 a | 24.55±2.82 a | 5.20 ±1.30 a | 6.33 ±1.75 a |

Treatments with the same letters in columns are not statistically significant different from each other.

difference was not recorded between okra plant sprayed with the extracts at 4, 5, 6, 8 and 9 WAP. However, there was significant difference in the severity of leaves recorded at 7 WAP. Significant difference was also recorded between *C. procera* and chemical insect controlled okra but *C. schweinfurthii* and *B. coccineus* were not statistically significant from each other. The highest severity was recorded at 9 WAP followed by 7 WAP which could be due to lack of rainfall in these periods.

Severity of leaves damage by *Podagrica* spp. is presented in Table 6. The least percentage leaves defoliation was observed from the plants treated with synthetic insecticide and *C. procera* treated plant recorded highest percentage of defoliation among the botanical insecticides. *C. schweinfurthii* and *B. coccineus* showed significantly promising effect in reducing severity of leaves damage. However, all botanical treated okra plants prevented the leaves from being severely defoliated as all the plants suffered below 33.32% defoliation at 9 WAP. In general term, leaves damage increased with the increasing age of okra plants.

*Phytochemical screening*

The result of phytochemical screening of crude leaf extracts revealed the presence of Triterpenoids, Steroids, Flavonoids, Phlobatanins, Saponins, Tannins, Cardiac glycoside and Anthraquinones in the plants (Table 7). Alkaloids and Anthraquinones were not detected in both *C. procera* and *C. schweinfurthii* while Triterpenoids and Phlobatanins were absent in *C. schweinfurthii.* The presence of these bioactive compounds in the plant materials is an indication that it may have some insecticidal potential against agriculturally important insect pest.

Table 7 - Phytochemical screening of crude aqueous extract of *C. procera*, *C. schweinfurthii* and *B. coccineus*

| Chemical constituents | *Calotrophus procera* | *Canarium schweinfurthii* | *Bryscocarpus coccineus* |
|---|---|---|---|
| Alkaloids | - | - | + |
| Terpenoids | + | - | + |
| Flavonoids | + | + | + |
| Anthraquinones | - | - | + |
| Tannins | + | + | + |
| Phlobatanins | + | - | + |
| Saponins | + | + | + |
| Cardiac glycosides | + | + | + |
| Steroids | + | + | + |

+ = Present, - = absent.

## 4. Discussion and Conclusions

Plants are rich sources of natural substances that can be utilized in the development of environmentally safe methods for insect pests control as veritable alternative to synthetic insecticide (Sadek, 2003**).** Moreover, growing awareness of health and environmental issues accompanied by the intensive use of chemical inputs has led to increased concerns and the need for alternate forms of crop protection in the world (Adesina, 2013).

The crude aqueous extracts evaluated in this study have been reported to contain insecticidal, termiticidal, antifeedant, ovacidal and larvacidal properties (Ahmed, 1993; Shaaya *et al*., 1977; David, 1989; Jahan *et al*., 1991; Abayeh *et al*., 1999; Abbassi *et al.*, 2003; Umsalama *et al*., 2006; Bakavathiappan *et al*., 2012; Katunku *et al.*, 2014; Nagawa *et al*., 2015; Okoli *et al*., 2016). The result of the study showed that the crude plant extracts were able to reduce insect population, but not significantly, compared to the synthetic insecticide, but were able to show some suppressing effect on the rate of feeding of the insects as the severity of the leaves damaged by the insects was not enough to cause significant reduction in yield compared to results obtained from okra plants sprayed with chemical.

The target insects are notorious for leaves defoliation owing to their biting and chewing mouthparts resulting in reduction of photosynthetic ability of the infested plants which invariably affect the plant growth and yield (Dent, 1999). The aqueous extracts were not effective at first two weeks of application. Synthetic insecticide recorded a higher efficacy compared to aqueous extract. This might be as a result of active ingredients of aqueous extracts being easily volatilized especially in the sun, thereby leading to their limited efficacy (Ware, 2000) and the delayed effect of aqueous extracts is reported to be one of the major problems of botanical insecticides (Oparaeke, 2006; Isman, 2008).

Since the beetles live and feed on the vegetative parts, any chemical that shows a remarkable efficacy against them must have contact toxicity, repellent or anti-feeding action. Several authors have reported that, the deleterious effects of crude plant extracts on insects are manifested in several ways, including toxicity (Hiremath *et al*., 1997) and feeding inhibition (Klepzig and Schlyter, 1999; Wheeler and Isman, 2001). Thus, reducing the level of beetle infestation and increasing okra pod carrying capacity; this confirmed the earlier report of Thirumalai *et al*. (2003)

who observed the effective reduction of mite population with application of neem seed kernel extract.

The reduction in *Podagrica* beetle population and increase in yield caused by the plant extracts treatment was comparable with those caused by synthetic insecticide. This supports the findings of Katunku *et al*. (2014) and those of Yusuf and Mohammed (2009) who reported that *C. schweinfurthii* and *Monordica balsamina* powder treatments were as effective as and comparable to permethrin and pirimiphos methyl in suppressing *C. maculatus* population and growth in cowpea storage.

The high rate of *Podagrica spp.* population reduction on exposure to the aqueous extracts treatments may be attributed to the chemical composition of the products. Plant extracts often consist of complex mixtures of bioactive constituents which may act as antifeedants, disturb insect growth, development and inhibit oviposition (Gerard and Ruf, 1991; Emimal Victoria, 2010). Thus, suggesting that most of the secondary metabolites such as terpenoids and alkaloids can be used as active insecticidal compounds that could be an effective alternative to synthetic insecticides for insect pest management. The toxicity of alkaloids was reported by Abbassi *et al.* (2003) and David (1989) reported that tannic acid acts as toxin and feeding deterrent to insects. Bernhoft (2010) reported that saponnins is found to affect the respiratory system of insects and causes emetic effect due to their detergent action. In the same vein, Philip *et al.* (2009) reported that plant products have repellent properties and toxic effect on the heart muscles in insects. Frazier (1986) reported that antifeedants can be found amongst all major classes of secondary metabolites (alkaloids, flavonoids, terpenoids and phenolics). Plant metabolites may produce toxic effects if ingested leading to rejection of the host plant (Russel and Lane, 1993).

The utilization of *C. schweinfurthii* and *B. coccineus* aqueous extracts had positive toxicity impact in decreasing the rate of *Podagrica spp.* infestation and also increased the yield of okra plant thus could serve as alternative to synthetic insecticide to protect okra plant against *Podagrica* spp. infestation to maximize the yield and income of the resource poor farmers.

**Acknowledgements**

The authors are grateful to Omowaye, Iyanuoluwa Daniel and Umaru, Muritala Oluwateru of Horticultural Technology Programme, Department of Crop, Soil and Pest Management Technology, Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria for the support received from them in collecting the plant materials used for the study.

**References**

ABAYEH O.J., ABDULRAZAQ A.K., OLAOGUN R., 1999 - *Quality characteristics of* Canarium schweinfurthii *Engl. Oil*. - Plant Foods Hum. Nutr., 54(1): 43-48.

ABBASSI K., ATAY-KADIRI Z., GHAOUT S., 2003 - *Biological effects of alkaloids extracted from three plants of Moroccan arid areas on the desert locust*. - Physiol. Entomol., 28: 232-236.

ADESINA J.M., 2013 - *Exploration of* Erythrina Excelsa *Baker and* Aneilema Beniniense *(P. Beauv.) Kunth aqueous extracts for the management of Flea beetles (*Podagrica Spp*) on Okra (*Abelmoschus Esculentus). - Nat. Sci*.,* 11(12): 210-215.

ADESINA J.M., AFOLABI L.A., 2014 - *Comparative bio-efficacy of aqueous extracts of* Loncarpous Cyanescens *and* Trema orientalis *against Flea beetle (*Podagrica spp.*) (*Coleoptera: Chrysomelidae*) infestation and yield of Okra*. - Int. J. Hortic., 4(2): 4-9.

AETIBA J.P.N., OSEKRE E.A., 2016 - *Management of insect pests of okra (*Abelmoschus esculentus *L. Moench) using oxymatrine-based Insecticide*. - Adv. Res., 6(1): 1-7.

AHMED B.I., YUSUF S.R., YUSUF A.U., ALIYU M., 2007 - *Comparative efficacy of different concentrations of some promising insecticides for the control of Podagrica spp. (*Coleoptera: Chrysomelidae*) on okra [*Abelmoschus esculentus *(L.) Moench]*. - Global J. Agric. Sci., 6: 31-34.

AHMED G.A., 1993 - *Preliminary investigation of the insecticidal potentialities of the Usher plant* Calotropis procera *Ait*. - M.Sc Thesis, University of Khartoun, Sudan.

ALAO F.O., ADEBAYO T.A., OLANIRAN O.A., AKANBI W.B., 2011 - *Preliminary evaluation of the insecticidal potential of organic compost extracts against insect pests of Okra [*Abemoschus esculentus *(L.) Moench]*. - Asian J. Plant Sci. Res., 1(3): 123-130.

ANTONIO B.D., 2009 - *Botanical pesticides: a part of sustainable agriculture in Babati District Tanzania*. - Bachelor's Thesis 15 ECTS, Södertörn University College, Sweden.

BAKAVATHIAPPAN G., BASKARAN S., PAVARAJ M., JEYAPARVATHI S., 2012 - *Effect of* Calotropis procera *leaf extract on* Spodoptera litura *(Fab.).* - J. Biopest., 5: 135-138.

BANSO A., NGBEDE J.E., 2006 - *Phytochemical screening and* in vitro *antifungal properties of* Fagara zanthoxyloides. - J. Food Agric. Environ., 4(3-4): 8-9.

BERNHOFT A., 2010 - *A brief review on bioactive compounds in plants. Bioactive compounds in plants - bene-*

*fits and risks for man and animals*. - The Norwegian Academy of Science and Letters, Proceedings, Oslo, 13-14 November, 2008, pp. 11-17.

DAVID N.K., 1989 - *Differential effect of taunic acid on two tree-feeing Lepidoptera: implication for theories of plant anti-herbivore chemistry*. - Oecol., 80(4): 507-512.

DENT D., 1999 - *Insect pest management*. - CABI Publishing, Wallingford, UK, pp. 432.

DUBEY N.K., BHAWANA S., ASHOK K., 2008 - *Current status of plant products as botanical pesticides in storage pest management.* - J. Biopest., 1(2): 182-186.

DUBEY N.K., KUMAR A., SINGH P., SHUKLA R., 2010 - *Exploitation of natural compounds in eco-friendly management of plant pests*, pp. 181-198. - In: GISI U., I. CHET, and M. GULLINO (eds.) *Recent developments in management of plant diseases*. Springer, Dordrecht, Germany, pp. 376.

EMIMAL VICTORIA E., 2010 - *Pest infestation on the biochemical modulation of* Adhatoda vasica. - J. Biopest., 3(2), 413-419.

FAJINMI A.A., FAJINMI O.B., 2010 - *Incidence of okra mosaic virus at different growth stages of okra plants [(*Abelmoschus esculentus *(L.) Moench] under tropical condition*. - J. Gen. Mol. Virol., 2(1): 28-31.

FASUNWON B.T., BANJO A.D., 2010 - *Seasonal population fluctuations of* Podagrica *species on okra plant (*Abelmoschus esculentus*)*. - Res. J. Agric. Biol. Sci., 6: 283-288.

FRAZIER J.L., 1986 - *The perception of plant allelochemicals that inhibit feeding*, pp. 1-42. - In: BRATTSTEN L.B., and S. AHMAD (eds.) *Molecular aspects of insect-plant associations*. Plenum Press, New York, USA, pp. 346.

GERARD P.J., RUF L.D., 1991 - *Screening of plants and plant extracts for repellence to* Tinea dubiella*, a major New Zealand wool pest*. - Proceedings of 44th N.Z. Weed and Pest Control Conference, pp. 205-208.

HIREMATH G.I., YOUNG-JOON A.H.N., KIM S.I., 1997 - *Insecticidal activity of Indian plant extracts against* Nilaparvata lugens *(*Homoptera: Delphacidae*).* - Appl. Entomol. Zool., 32(1): 159-166.

ISMAN M.B., 2008 - *Botanical insecticides: for richer, for poorer*. - Pest Manag. Sci., 64(1): 8-11.

JAHAN S., MAMAN A., KHAN A.R., 1991 - *Insecticidal effects of* Akanda Calotropis procera *on* Tribolium confusum *Duval (*Coleoptera: Tenebrionidae*).* - Bangladesh J. Zool., 19: 261-268.

KATUNKU D., OGUNWOLU E.O., UKWELA M.U., 2014 - *Contact toxicity of* Canarium schweinfurthii *Engl. tissues against* Callosobruchus maculatus *in stored bambara groundnut*. - Int. J. Agron. Agric. Res., 5(5): 20-28.

KEDAR S.C., KUMARANAG K.M., BHUJBAL D.S., THODSARE N.H., 2014 - *Insect pest of Okra and their management*. - Popular Kheti, 2(3): 112-119.

KLEPZIG K.D., SCHLYTER F., 1999 - *Laboratory evaluation of plant-derived antifeedants against the pine weevil* Hylobius abietis *(*Coleoptera: Curculionidae*).* - J. Econ. Entomol., 92(3): 644-650.

NAGAWA C., BÖHMDORFER S., ROSENAU T., 2015 - *Chemical composition and anti-termitic activity of essential oil from* Canarium schweinfurthii *(Engl.).* - Ind. Crops Prod., 71: 75-79.

NGBEDE J., YAKUBU R.A., NYAM D.A., 2008 - *Phytochemical screening for active compounds in* Canarium schweinfurthii *(Atile) leaves from Jos North, Plateau State Nigeria*. - Res. J. Biol. Sci., 3(9): 1076-1078.

OBENG-OFORI D., SACKEY J., 2003 - *Field evaluation of non-synthetic insecticides for the management of insect pests of okra* Abelmoschus esculentus *(L.) Moench in Ghana*. - Ethiopian J. Sci., 26: 145-150.

ODEBIYI A., SOFOWORA A.E., 1978 - *Phytochemical screening of Nigerian medicinal plants*. - Lloydia, 41(3): 234-246.

ODEBIYI J.A., 1980 - *Relative abundance and seasonal occurrence of* Podagrica *spp. (*Coleoptera: Chrysomelidae*) on okra in Southwestern Nigeria*. - African J. Agric. Sci., 6: 83-84.

OKOLI B.J., NDUKWE G.I., AYO R.G., HABILA J.D., 2016 - *Inhibition of the developmental stages of* Ascaris suum *and antimicrobial activity of 3β-Hydroxylolean-12,18-diene isolated from the aerial parts of* Canarium schweinfurthii *(Engl.).* - Am. Chem. Sci. J., 11(3): 1-11.

OPARAEKE A.M., 2006 - *The potential for controlling* Maruca vitrata *Fab. and* Clavigralla tomentosicollis *Stal. using different concentrations and spraying schedules of* Syzigium aromaticum *(L.) Merr and Perr on cowpea plants*. - J. Plant Sci., 1: 132-137.

PHILIP K., MALEK S.N.A., SANI W., SHIN S.K., KUMAR S., LAI H.S., SERM L.G., RAHMAN S.N.S.A., 2009 - *Antimicrobial activity of some medicinal plants from Malaysia*. - Am J Appl. Sci., 6(8): 1613-1617.

RUSSELL G.B., LANE G.A., 1993 - *Insect antifeedants - a New Zealand perspective*. - Proc. 46th N.Z. Plant Prot. Conf., pp. 179-186.

SADEK M.M., 2003 - *Antifeedant and toxic activity of* Adhatoda vasica *leaf extract against* Spodoptera littoralis *(*Lepidoptera: Noctuidae*).* - J. Appl. Entomol., 127(7): 396-404.

SHAAYA E., KOSTJUKOVYSKI M., EIBERY J., SUKPRAKARN C., 1997 - *Plant oils as fumigants and contact insecticides for the control of stored-product insect*. - J. Stored Prod. Res., 33: 7-15.

SOFOWORA A., 1982 - *Medicinal plants and traditional medicine in Africa*. - John Wiley and Sons., pp. 142-146.

THIRUMALAI THEVAN P.S., SRINIVASAN T.R., KUAMAR N., MANOHARAN T., MUTHUKRISHNAN N., 2003 - *Bio-efficacy of botanicals against coconut eriophyid mite,* Aceria guerreronis *Keifer*. - Nat. Symp. on BioManagement of Insect Pests, Tamil Nadu Agricultural University, Coimbatore, India, 29-31 March, pp. 107.

UMSALAMA A.M.A., SHI Z., NABIL H.H.B., 2006 - *Evaluation and insecticidal potentialities of aquoues extracts from* Calotropis procera *Ait against* Henosepilachna elaterii

*Rossi.* - J. Applied Science, 6(11): 2466-2470.

WARE G.W., 2000 - *The pesticide book*. - Thomson Publications, Fresno, California, USA, pp. 197.

WHEELER D.A., ISMAN M.B., 2001 - *Antifeedant and toxic activity of* Trichilia americana *extract against the larvae of* Spodoptera litura. - Entomol. Exp. Appl., 98(1): 9-16.

WILLIAMSON E.M., OKPAKO D.G., EVAN F.J., 1996 - *Pharmacological methods in phytotherapy research. Vol. 1. Selection, preparation and pharmacological evaluation of plant materials*. - John Wiley and Sons Ltd. Chichester, UK, pp. 9-13.

YUSUF S.R., MOHAMMED Y.S., 2009 - *Potentials of bitter mellon* Monordica balsamina *(L.) and asthma plant [*Mitracarpus villosus *(L.) Walp] against cowpea bruchid (*Callosobruchus maculatus *(F.) damage*. - Savanah J. Agric., 4: 54-61.

Adv. Hort. Sci., 2018 32(1): 79-92 DOI: 10.13128/ahs-20646

AHS
Advances in Horticultural Science

# Cotton flowering behavior, fiber traits and gene expression under water-shortage stress

**D. Jawdat [1] (*), A.W. Allaf [2], N. Taher [1], A. Al-Zier [2], N. Morsel [1], Z. Ajii [2], B. Al-Safadi [1]**

[1] *Department of Molecular Biology and Biotechnology, Atomic Energy Commission, PO Box 6091, Damascus, Syria.*

[2] *Department of Chemistry, Atomic Energy Commission, PO Box 6091, Damascus, Syria.*

*Key words*: cotton, fiber quality, flowering, FTIR, gene expression, TGA, XRD.



**Abstract: Two accredited cotton (*Gossypium hirsutum* L.) cultivars (Aleppo 118 and Deir Al-Zour 22) have been investigated to assess physiological, morphological, and molecular responses under water shortage conditions. Both cultivars have shown early flowering. However, higher percentage of treated 'Aleppo 118' plants kept flowering towards the end of the growing season compared to treated 'Deir Al-Zour 22' plants. Both cultivars kept consistent micronaire and cohesion under normal and water shortage conditions. The cultivar Aleppo 118 displayed more consistent fiber quality between control and treated plants, while 'Deir Al-Zour 22' showed variation in fiber length and strength between control and treated plants. Results have demonstrated an increase in fold expression of *DREB1A*, *TPS* and *HSPCB* genes in the flowering stage of treated plants compared to the controls. Results also showed a continuous activation of *DREB1A* gene in the two critical growing stages of a cotton life cycle, flowering and boll development in treated plants of 'Deir Al-Zour 22'. This work illustrates the different responses of two cotton cultivars under water shortage stress and its impact on flowering and fiber traits.**

**(*) Corresponding author:**
ascientific2@aec.org.sy

**Citation:**
JAWDAT D., ALLAF A.W., TAHER N., AL-ZIER A., MORSEL N., AJII Z., AL-SAFADI B., 2018 - *Cotton flowering behavior, fiber traits and gene expression under water-shortage stress.* - Adv. Hort. Sci., 32(1): 79-92



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 10 May 2017
Accepted for publication 12 January 2018

## 1. Introduction

The supply of groundwater in agriculture is reduced due to intersecting environmental, industrial and domestic sectors. Groundwater-dependent agro-economies are mostly affected by drought stress, water deficits and aquifer depletion, specifically in the arid and semi-arid regions. Drought is considered a major constraint to crop productivity and yield stability around the world. It is therefore, a major challenge to farmers in drought subjected regions. Water saving and stable crop production are the main challenges that urge researchers to develop new cultivars and irrigation strategies for a sustainable use of water in agriculture.

Developing new cultivars that tolerate water stress has been assisted

and supported by wide range of strategies and procedures that enable the selection of candidate parents and an efficient screening of targeted progenies. Genomics, marker-assisted selection, the manipulation of QTLs using genetic engineering and transcriptomics can contribute in the release of improved, drought-tolerant cultivars (Tuberosa and Salvi, 2006). The metabolomics-assisted breeding is another candidate approach for the development of crops with increased tolerance to abiotic stresses (Fernie and Schauer, 2009). Improving drought tolerance in crops requires understanding the biochemical responses under water deficit (Reddy *et al.*, 2004) and the characterization of drought patterns in the growing regions along with the physio-morphological traits of these crops under such environments (Fukai and Cooper, 1995; Khan *et al.*, 2010). Plants have evolved their drought-adaptive strategies (Meyre *et al.*, 2001), between drought escape (early flowering) and drought tolerance. Comprehending the flowering pattern of each crop under stressed and non-stressed environments is a key to which drought-adaptive strategy the crop plant follows. Early flowering and maturity can help in saving groundwater for irrigation and can help in avoiding early rainfall that usually affects the harvest of certain crops such as cotton (Jawdat *et al.*, 2012). Therefore, a vigilant assessment of crop responses to alteration in irrigation regimes is needed. At any rate, amendments and management of irrigation and water saving methods has a great potential in saving water sources in the arid and semi-arid regions (Oweis *et al.*, 2011; Ünlü *et al.*, 2011).

Cotton is a major cash oilseed and fiber crop with a world production estimated to be 25.01 million tonnes in 2013-2014 as announced at the official website of the International Cotton Advisory Committee (ICAC) (https://www.icac.org). The widely famous, allotetraploid *Gossypium hirsutum* L. has a distinct growth manner and can be useful in monitoring growth and developmental changes under diverse conditions (Jawdat *et al.*, 2012). Cotton is grown in around 70 countries where the irrigated system dominates in arid regions; and is used to supplement rainfall in the humid regions to ensure yield stability. Seed cotton production in Syria (mostly semi-arid where cotton is irrigated) was around one million tonnes in 2000. A decrease in production from around 670 K tonnes in 2009 to around 472 K tonnes in 2010 and then up to about 671 K tonnes in 2011 in the same cultivation area (~200 K hectares), was attributed to the drought wave that hit the country in 2010. A rapid decline in production was recorded between 2011 and 2014, from around 670 to around 162 K tonnes respectively. This was concurrent with the unfortunate events taking place in Syria.

The principal objective of this paper is to assess physiological, morphological, molecular, and biochemical responses of two accredited cotton cultivars in Syria under deficit water regime, in benefits of future breeding programs.

## 2. Materials and Methods

*Plant materials*

Plant material consisted of cotton seeds of two *G. hirsutum* L. accredited local cultivars: Aleppo 118 [Aleppo 40 (Aleppo 1 x Acala SJ1) x American cultivar BW 76-31] and Deir Al-Zour 22 (a selected line from Deltapine 41).

*Drought stress treatment*

Seeds were sown in rows, where the distance between rows and plants were kept at 75 and 25 cm respectively. Experimental plots were designed according to a randomized complete block design (RCBD) with each treatment is replicated once in each block. The experiment site was located in Doubaya in Damascus country side (980 m above sea level, 33° 29′ 36′ N and 36° 04′ 57′ E). The experiment consists of two irrigation treatments (control with full irrigation and treated with 50% of control irrigation), two cultivars (Aleppo 118 and Deir Al Zour 22) and three replicates per treatment. The soil (total N: 0.08%, available P: 12 ppm, K: 1.30 Meq/100 g, and organic matter: 1.19%) was prepared by tractor ploughing deep for 45 cm, followed by two surface ploughings for 15 cm deep. Fertilization was conducted following the Cotton Research Administration (CRA) guidelines and recommendations. Nitrogen fertilizer as urea 46% [$CO(NH_2)_2$] was applied at a rate of 415 Kg/ha in four unequally split applications, 20% before sowing, 40% 30 days after sowing, 20% at floral bud emergence, and 20% at bolls initiation phase. Phosphorus fertilizer [$Ca(H_2PO_4)_2$] was applied at a rate of 130 Kg/ha before sowing. Potassium fertilizer was applied at the rate of 170 Kg/ha in the form of potassium sulphate ($K_2SO_4$). Drip irrigation was applied at 250 $m^3$/ha, at first, for both control and treated blocks. Consequent irrigations were applied during the growing season of cultivated plants once a week at 250 $m^3$/ha (full irrigation) in control blocks, and once in every 10 days at 125 $m^3$/ha (deficit irriga-

tion; receiving water at 50% of control treatment) in the treated blocks. Except for one week where temperatures reached around 45 degrees in the afternoon, we had to irrigate both control and treatment blocks with emergency full irrigation. The distance between emitters was 25 cm and the emitter discharge was 8 L/h. Thinning of plants was conducted around 4 weeks of germination.

*Physiological data*

*Chlorophyll a and b content.* Leaves samples (100 mg/sample) were each immersed in 4 ml of 95% acetone and incubated at 6-8°C for 24 hrs. Chlorophyll a and b concentration was determined spectrophotometrically by measuring the absorbance (optical density-OD) at 662 and 644 nm respectively. The concentrations of Chlorophyll a and Chlorophyll b (µg. $g^{-1}$ FW) in leaf tissues were calculated using the following equations (Cha-Um *et al.*, 2006):

$$\text{Chlorophyll a} = 9.784 * D_{662} - 0.99 * D_{644}$$

$$\text{Chlorophyll b} = 21.426 * D_{644} - 4.65 * D_{662}$$

Where Di is an optical density at the wavelength i.

*Osmotic potential OP) and osmotic adjustment (OA).* Osmometer readings in mOsm/kg, were taken using the freezing point osmometer, the Micro-Osmoette™ (Precision systems Inc., USA). Frozen 100 mg of leaf samples in liquid nitrogen were ground (a pool of three leaves per treatment). Two milliliters of autoclaved d.d. water were added to each sample and samples were vortexed briefly and centrifuged for one minute at 13000 rpm. Fifty microliters of the supernatant were used for each reading. The osmotic potential Ψs was calculated using the following formula:

$$\text{OP (MPa)} = -\{R * T * \text{osmometer reading}\}/1000$$

where R is the gas constant (0.008314), and T is the laboratory temperature in Kelvin (298). The osmotic adjustment (OA) was calculated as the difference between the osmotic potential of non-stressed plants and the water stressed plants.

*Morphological data*

Twenty plants of each replicate were randomly tagged and flowering behavior data for days after planting (DAP) were recorded such as: first floral bud emergence, plant height at first flower, and nodes above white flower (NAWF). The percentage of plants bearing flowers and plants bearing floral buds were recorded towards end of season. End of season data also included: plant height, root depth (randomly selected plants from each replicate were carefully pulled out of ground), and number of secondary roots.

*Physical and chemical fiber properties*

*Fiber quality testing.* Thirty bolls of each replicate were picked and sent to the fiber quality lab at the Cotton Research Administration (CRA) for testing: micronaire, fiber length, fiber strength, fiber cohesion, and ginning percentage. CRA fiber quality lab conducts its testing according to the international testing standards with a temperature of 22±2°C and relative humidity of 64%±4%. The lab uses the following instruments: Micronaire 775, Digital Fibrograph, Pressley tester and Stelometer for fiber testing.

*Fourier Transform Infrared (FTIR) acquisition spectra of cotton fiber*

FTIR was conducted to study the chemical variance, if any, between fibers from control and treated plants of the two studied cultivars. The infrared spectra were recorded on Nicolet 6700 in the range 4000 to 400 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ at 32 scans as direct measurements of the cotton fibers (three replicates per treatment) samples using universal attenuated total reflectance technique FTIR-ATR with KRS-5 crystals. The instrument is equipped with DTGS detector and KBr beam splitter. The operating system used was the OMNIC software (Version 7.3, Thermo Nicolet, USA). The infrared spectra of fiber samples for control and treated plants of Aleppo 118 and Deir Al Zour 22 cultivars were recorded. The recorded spectra were repeated three times for each investigated sample.

*Measurement of crystallinity by conventional X-Ray powder diffractometry (XRD)*

XRD was applied to determine the crystallinity of fiber samples from control and treated plants of 'Aleppo 118' and 'Deir Al-Zour 22' samples. X-ray powder diffraction patterns were obtained on a STOE transmission Stadi-P diffractometer with monochromatic Cu $K_{\alpha 1}$ radiation ($\lambda$ = 1.5406 Å) selected using an incident-beam curved-crystal germanium Ge (111) monochromator, with the STOE transmission geometry (horizontal set-up) and a linear position-sensitive detector (PSD).

The determination of crystallinity of the samples was performed with the program *WinXPOW* (Stoe and Cie, 1999) using single-sample method. This method requires defining the portions of the diagram due to air scatter and inelastic scattering, amorphous scattering and Bragg or crystal scattering. If $I_c(2q)$ and $I_a(2q)$ are known for the whole diagram, the crystallinity index is calculated from the relation:

$$x_c = (\sum I_c(2\theta) / (LP \cdot F \cdot T) / (\sum [(I_c(2\theta) / T + I_a(2\theta)] / (LP \cdot F) \qquad \textit{Eq. 1}$$

The summing is over Bragg angle (*2q*). The Lorentz-Polarization factor *LP*, the average form factor *F* and the temperature factor *T* are all 2q-dependent functions:

$LP(2\theta) = 1 + \cos^2(2\theta) / (\sin^2(2\theta)\cos\theta$ *Eq. 2*

$F(2\theta) = \sum f\ (n, 2\theta)$ *Eq. 3*

$T(2\theta) = \exp(-2B \sin^2 2\theta / \lambda^2)$ *Eq. 4*

The summing in Eq. 3 is over all atoms in the formula unit. The sample's overall formula and average temperature factor (*B*) have to be known in order to be able to apply this method. It's noteworthy to mention that sample absorption is always neglected; therefore, this method works well for transmission data of organic samples within ± 3% error of the true crystallinity. The formula of cellulose ($C_6H_{12}O_5$) and *B* = 4.0 Å$^2$ was used for our cotton fiber samples.

*Thermogravimetry analysis (TGA) and differential scanning calorimetry (DSC)*

The dynamic weight loss of fiber samples as a function of increasing temperature tests were conducted using a Mettler instrument (TG50). The tests were carried out in a nitrogen atmosphere, purged (30 ml/min) using sample weights of 10-15 mg at a heating rate of 10°C/min. The resolution of the balance is given, as 1 microgram for weights less than 100 milligrams, and the temperature precision of the instrument is ± 2°C.

DSC was used to determine the glass transition temperatures of the prepared samples. A Mettler instrument (DSC20) was utilized to record the DSC spectra. All samples were tested in aluminum pans at a heating rate of 10°C/min over a temperature range from room temperature to 400°C. The precision of the used instrument is ± 0.2°C.

*Scanning electron microscopy (SEM)*

Scanning electron microscopy (VEGAII XMU, TESCAN, Czech Republic) with an accelerating voltage of 30 Kv, was operated to capture SEM images of cotton fibers coming from control and treated plants of both cultivars.

*Gene expression analysis*

*RNA isolation and cDNA synthesis.* Total RNA was isolated from leaves of cotton plants using the modified hot borate method (Jawdat and Karajoli, 2012). Quality of total RNA was tested by agarose-formaldehyde gel electrophoresis using standard protocols. The iScript™ Select cDNA Synthesis kit (BioRad, USA) was used to obtain first strand cDNA starting from 1 µg total RNA following the manufacturer's protocol. The cDNA was diluted 4X and stored at -20°C.

*Relative quantification of gene expression*

The cDNA samples (control and treated) were used as a template to quantify target genes (*DREB1A*, *TPS* and *HSPCB*) expression level. Real-Time PCR was performed in two replicates in a 25 µl of a reaction mixture composed of 12.5 µl IQ SYBR super mix (BioRad, USA), 1 µl of cDNA, 1 µl of each of the forward and reverse primer (10 µM), and 9.5 µl of d.d. water.

The quantification of mRNA levels was normalized with the level of mRNA for *GhEF1α5* (Artico *et al.,* 2010). The relative expression (fold expression) was calculated using the $-\Delta\Delta C_t$ method as follows:

$$2^{-\Delta\Delta Ct} = 2^{-[(Ct\ treated\ target\ gene - Ct\ treated\ reference\ gene) - (Ct\ untreated\ target\ gene - Ct\ untreated\ reference\ gene)]}$$

Specific target and reference gene primers are listed in Table 1.

## 3. Results

Physiological, morphological, molecular and chemical data were recorded to identify main changes in two cash cultivars of cotton. The physiological and molecular analysis were carried out on leaf material from control and treated plants of 'Aleppo 118' and 'Deir Al-Zour 22', harvested 45 DAP

Table 1 - Target and reference genes accession numbers and primers sequences

| Gene | GenBank accession No. | Forward and reverse primers (5'-3') |
|---|---|---|
| Dehydration responsive element binding gene (DREB1A) | AY321150.3 | F-AGCTATAGCACTGAGAGGGAAG |
| | | R-GCTTCTTCGTCCAAGTAAAACC |
| Trehalose-6-phosphate-synthase gene (TPS) | AY628139.1 | F-TTCACTACATGCTGCCCATGTG |
| | | R-GGCTGTGGAGGAAAAAACCAAG |
| Heat-shock protein calmodulin binding gene (HSPCB) | AY819767.1 | F-CTCCTTGAATGTATTTACTGCC |
| | | R-GTGCGTCCTCTAGTGTCTTT |
| Elongation Factor 1-alphagene (Ef1α5) | DQ174254 | F-TCCCCATCTCTGGTTTTGAG |
| | | R-CTTGGGCTCATTGATCTGGGT |

in the course of floral bud initiation stage.

*Chlorophyll a and b content and osmotic potential*

The chlorophyll a content showed a non-significant decreasing trend in treated plants compared to control plants in both cultivars. However, the chlorophyll b content showed a small increment in the treated plants. The chlorophyll a/b ratio showed a reduction in treated plants of both cultivars. A larger reduction (18%) was observed in treated plants of 'Deir Al-Zour 22' compared to the small reduction (4%) in treated plants of 'Aleppo 118' (Fig. 1 inset graph). A non-significant drop in the osmotic potential of leaves in treated plants was observed in both cultivars and the osmotic adjustment was higher (0.016 MPa) in 'Aleppo 118' compared to 'Deir Al-Zour 22' (0.006 MPa) (Fig. 1).

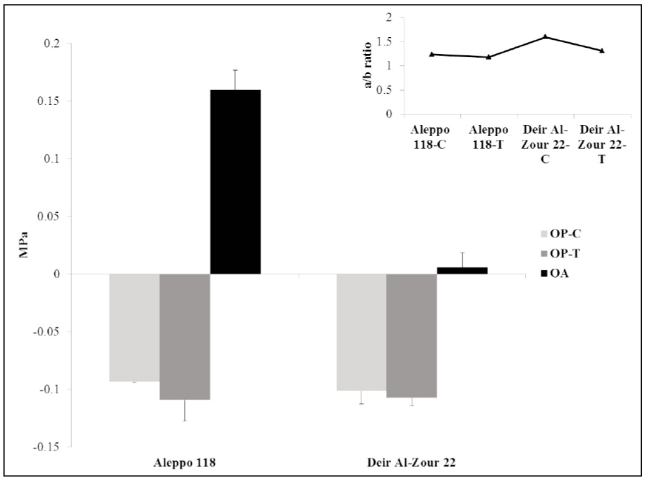



Fig. 1 - The inset graph is chlorophyll a/b ratio in leaves of control (C) and treated (T) 'Aleppo 118' and 'Deir-Al Zour 22' plants. Main graph represents osmotic potential and osmotic adjustment in leaves of control and treated 'Aleppo 118' and 'Deir Al-Zour 22' plants. Plants were under drip irrigation system, where the control plants were given full irrigation each week, and treated plants have been given deficit irrigation each 10 days. Data represent means and standard error of three replications.

*Plant morphology*

Early floral bud emergence was observed in both treated cultivars compared to control plants (Fig. 2 inset graph I). The control plants of Aleppo 118 cultivars flowered earlier than control plants of 'Deir Al-Zour 22'. NAWF counts, another indicator of flowering earliness, was also recorded in the third week of first flower emergence (Fig. 2 inset graph II). Recorded NAWF counts showed a significant decrease in treated plants of both cultivars. A final record of plants with flowers, and plants with floral bud percentages were also taken five months after planting towards the end of season (Fig. 2). End of season flowering counts have shown 'Aleppo 118' tendency to keep initiating floral buds (~ 21%) in treated plants compared to control plants (5.6%), and produce fully opened flowers (~ 31%) in treated compared to control plants (~ 2%). However, a similar but less potent flowering counts pattern was observed in Deir Al-Zour 22 cultivar.

End of season measurements have also included plant stem height, taproot depth and number of secondary roots (Table 2). Treated plants in both cultivars showed a slight increase in plant stem height and minor decrease in root depth. However, non-significant increase in number of secondary roots was observed in treated plants of both cultivars.

*Physical and chemical fiber properties*

*Fiber quality testing.* The two tested cultivars have presented slightly different behavior in terms of fiber properties. Seed cotton yield showed no significant difference between treated and control plants in each cultivar. The cultivar Deir Al-Zour 22 showed a significant increase in both fiber length and strength in the treated plants compared to control ones. Fiber

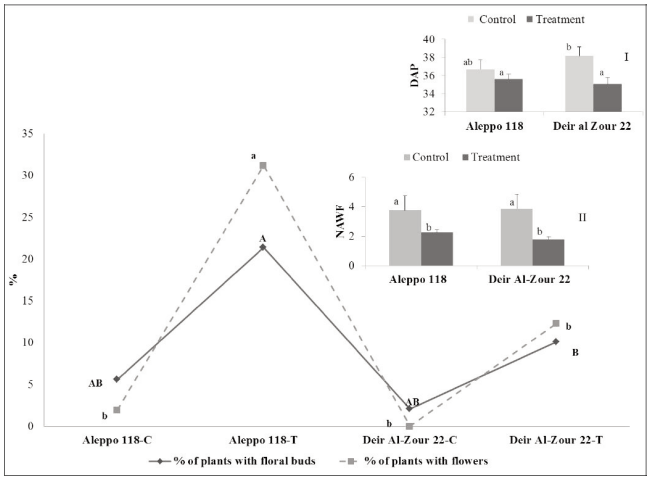



Fig. 2 - The inset graph-I shows floral buds emergence in days after planting (DAP) in control and treated 'Aleppo 118' and 'Deir Al-Zour 22' plants. The inset graph-II represents NAWF counts in control and treated plants of both cultivars. Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Columns sharing a letter are not significantly different. Data represent means and standard error of three replications. The Original graph is the percentages of plants with flowers and plants with floral buds of both control and treated Aleppo 118 and Deir Al-Zour 22 cultivars, five months after planting. Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Line markers sharing a letter are not significantly different (capital letters for plants with floral buds and small letters for plants with flowers.

Table 2 - End of season measurements of plant stem height, taproot depth and number of secondary roots in control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars

| Cultivar | Plant height (cm) | | Root depth (cm) | | No. secondary roots | |
|---|---|---|---|---|---|---|
| | Control | Treatment | Control | Treatment | Control | Treatment |
| Aleppo 118 | 99.3±3.5 ab | 105.9±5.5 a | 19.8±2.2 | 17.7±2.1 | 10.4±0.8 AB | 13.1±0.8 A |
| Deir Al-Zour 22 | 80.3±3.8 c | 87.6±5.1 bc | 18.3±2.8 | 17.0±0.7 | 9.9±0.9 B | 11.4±1.1 AB |

Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Numbers in rows and columns sharing a letter in each block (Plant height and No. secondary roots) are not significantly different. Data represent means and standard error of three replications.

elongation showed no significant difference between control and treated plants in each cultivar. Furthermore, no significant difference was recorded in the cultivar Aleppo 118. Two fiber properties, fiber cohesion and micronaire, exhibited no significant difference between cultivars and between treatments. A significant increase in fiber maturity was observed in the treated plants of Aleppo 118 cultivar, whereas, a non-significant increase was recorded in treated plants of 'Deir Al-Zour 22' (Fig. 3).

*FTIR-ATR spectra acquisition of the cotton fiber.* The FTIR-ATR spectra of fiber samples from control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars were recorded. The spectra showed a typical characteristic reflectance bands for common cotton fiber substances. The spectra of fiber samples from control and treated 'Aleppo 118' plants were aligned for comparison and showed minimum variation (Fig. 4 A). The spectra of fiber from 'Aleppo 118' control plants showed 15 distinctive bands centered at 3310, 2890, 1740, 1605, 1429, 1372, 1315, 1203, 1160, 1103, 1055, 1020, 675, 554 and 431 $cm^{-1}$. The vibration at 1429 $cm^{-1}$ is designated and assigned as a "crystalline" absorption band of the fiber (Abidi *et al.*, 2014). The band at 1372 $cm^{-1}$ vibration is assigned to C-H bending, and it may be most suitable for indicating cellulose crystallinity associated with the band at 2890 $cm^{-1}$. The spectra of control and treated samples were highly similar except for the band at 1740 $cm^{-1}$ which was absent in the treated sample.

Similar spectra range was observed in Deir Al-Zour 22 cultivar. However, few noticeable new bands with distinctive structures were observed in the treated sample at 2890, 1740, and 1605 $cm^{-1}$. The band centered at 2890 $cm^{-1}$ contains two prominent peaks vibrations at 2910 and 2847 $cm^{-1}$, which are assigned to $CH_2$ asymmetrical and symmetrical stretching,

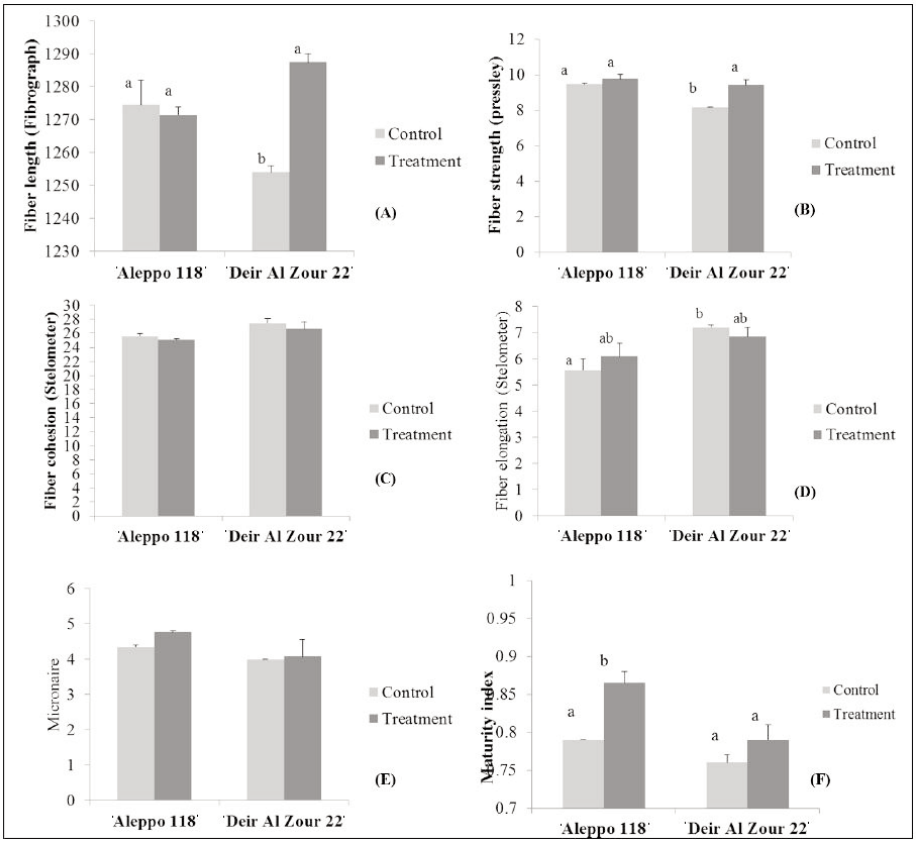



Fig. 3 - Fiber properties of control and treated Aleppo 118 and Deir Al-Zour 22 cultivars. Fiber length (A), fiber strength (B), fiber cohesion (C), fiber elongation (D), micronaire (E), and maturity index (F). Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Columns sharing a letter are not significantly different. Data represent means and standard error of three replications.

respectively. As for the second band at 1740 $cm^{-1}$, which is assigned to a carboxylic ester group (C=O stretching), a clearer structure is noticed in the treated sample in comparison with the control. Another distinctive banding pattern is observed at 1605 $cm^{-1}$ (adsorbed water), which is neither seen in the treated Aleppo 118 cultivar and nor observed in Deir Al-Zour 22 cultivar control sample. This band contains two prominent peaks vibrations at 1577 and 1540 $cm^{-1}$ (Fig. 4 B).

*Measurement of crystallinity by conventional x-ray powder diffractometry.* Fiber analysis using the XRD procedure showed a typical cellulose pattern in both control and treated plants for each cultivar (Fig. 5). The amount of crystalline cellulose in studied samples was between 66-75% (Table 3). As seen in Table 3, there was a minor difference between the CI values of the treated and control 'Deir Al-Zour 22' samples. However, there is no variation between control and treated samples of Aleppo 118 cultivar.

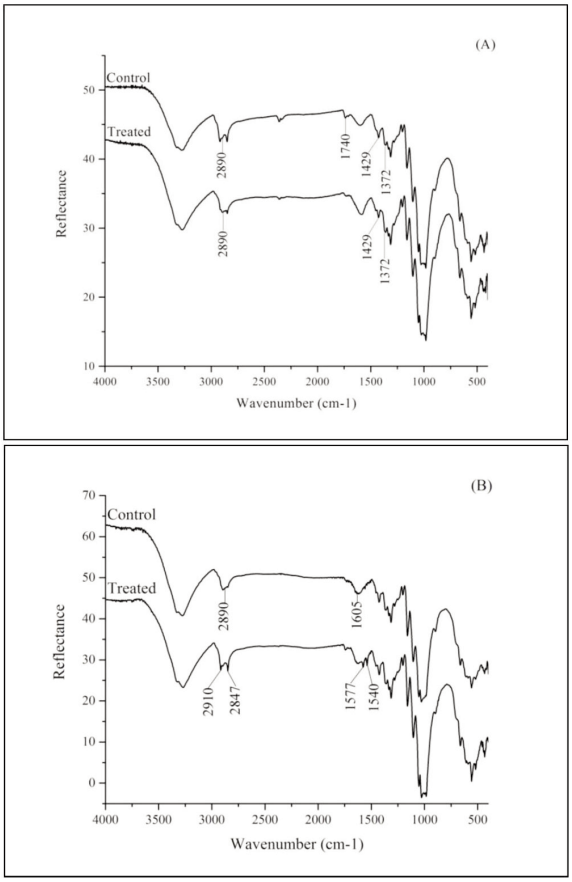



Fig. 4 - FTIR-ATR spectra of the cotton fibers obtained from (A) 'Aleppo 118' control and treated plants and (B) 'Deir Al-Zour 22' control and treated at room temperature in the range 400-4000 $cm^{-1}$. The recorded spectra were repeated three times and showed a typical and characteristic absorption bands for common cotton fibers substances.

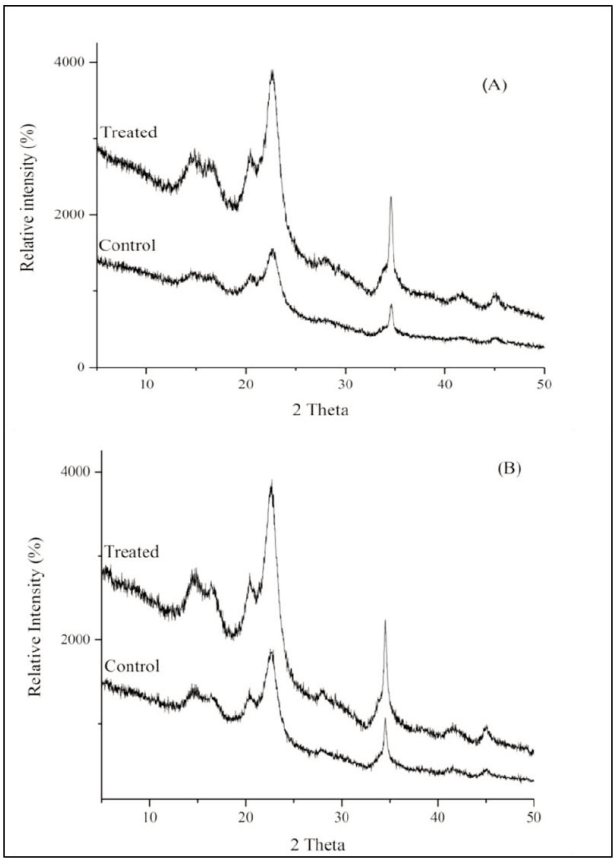



Fig. 5 - X-ray powder diffraction patterns of fibers in control and treated Aleppo 118 and Deir Al-Zour 22 cultivars.

Table 3 - Fiber crystalline index in control and treated Aleppo 118 and Deir Al-Zour 22 cultivars

| Cultivar | Crystallinity Index (%) | |
|---|---|---|
| | Control | Treatment |
| Aleppo 118 | 66.5±3.0 | 66.8±3.0 |
| Deir Al-Zour 22 | 73.7±3.0 | 68.5±3.0 |

*Thermogravimetry analysis (TGA) and differential scanning calorimetry (DSC).* Typical dynamic TGA thermograms of the studied samples have been recorded and are shown in figure 6 A. The TGA thermograms show a slight decrease in the weight and one significant step at high temperature.

Differential scanning calorimetry (DSC) was used to locate a possible glass transition temperature ($T_g$) of the samples. The glass transition temperature could not be observed in all DSC thermograms. The endothermic peak at around 100°C could be due to evaporation of adsorbed water (Fig. 6 B).

*Scanning electron microscopy (SEM).* SEM images reveal the longitudinal morphology of fibers coming from control and treated plants of Aleppo 118 and Deir Al-Zour 22 cultivars under three magnifications

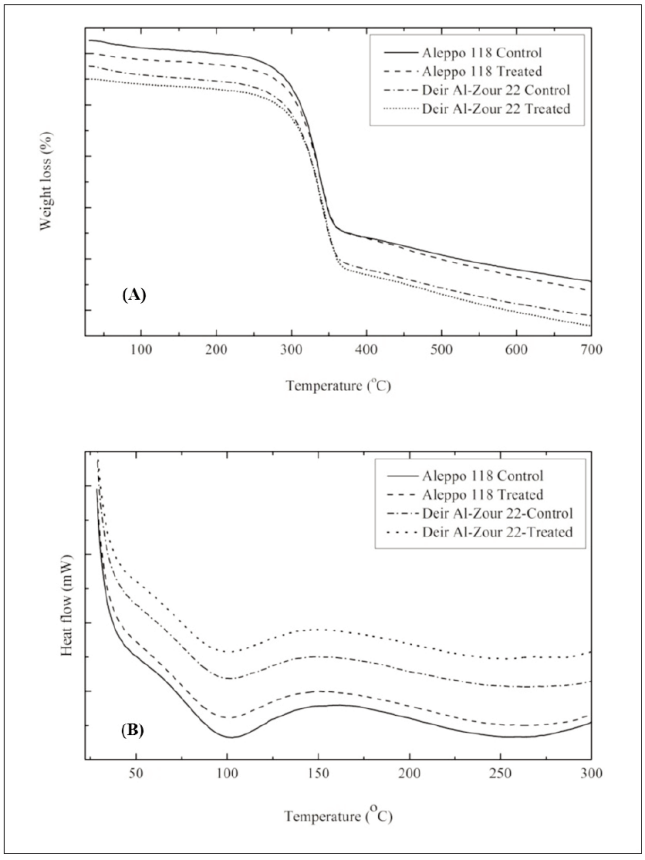



Fig. 6 - (A) TGA thermograms of cotton fibers in presence of nitrogen. (B) DSC thermograms of cotton fibers in presence of nitrogen.

(1.00 kx, 3.00 kx and 20.00 kx) (Fig. 7). Images of the two cultivars show the ribbon fiber shape rolled in a helicoid manner, a typical cotton morphological feature. The spiral and twisting fiber nature is present in both cultivars under both conditions.

*Expression analysis of DREB1A, TPS and HSPCB drought-related genes*

Fold expression of three drought-related genes in cotton (*DREB1A*, *TPS* and *HSPCB*) was analyzed, assisted by *GhEF1α5* gene expression for normalization (Fig. 8). Leaf samples of control and treated plants of both cultivars were harvested at two points (stages), S1 and S2. Where S1 represents leaf material harvested at 40 DAP (during floral bud to flower transition stage) and S2 represents leaf material harvested at 60 DAP (bolls opening and maturation). Gene expression analysis showed an activation of the studied genes in treated plants compared to the controls of both cultivars during S1. The S2 stage, experienced a drop in genes activity in both treated cultivars. Analysis showed a major significant drop in gene activity (*HSPCB* and *TPS*) in treated 'Deir Al-Zour 22' plants (S2) compared to samples of the earlier stage (S1). Exceptionally, *DREB 1A* gene showed a slight decrease in gene expression keeping its high activity in treated plants ~4 fold higher than the con-

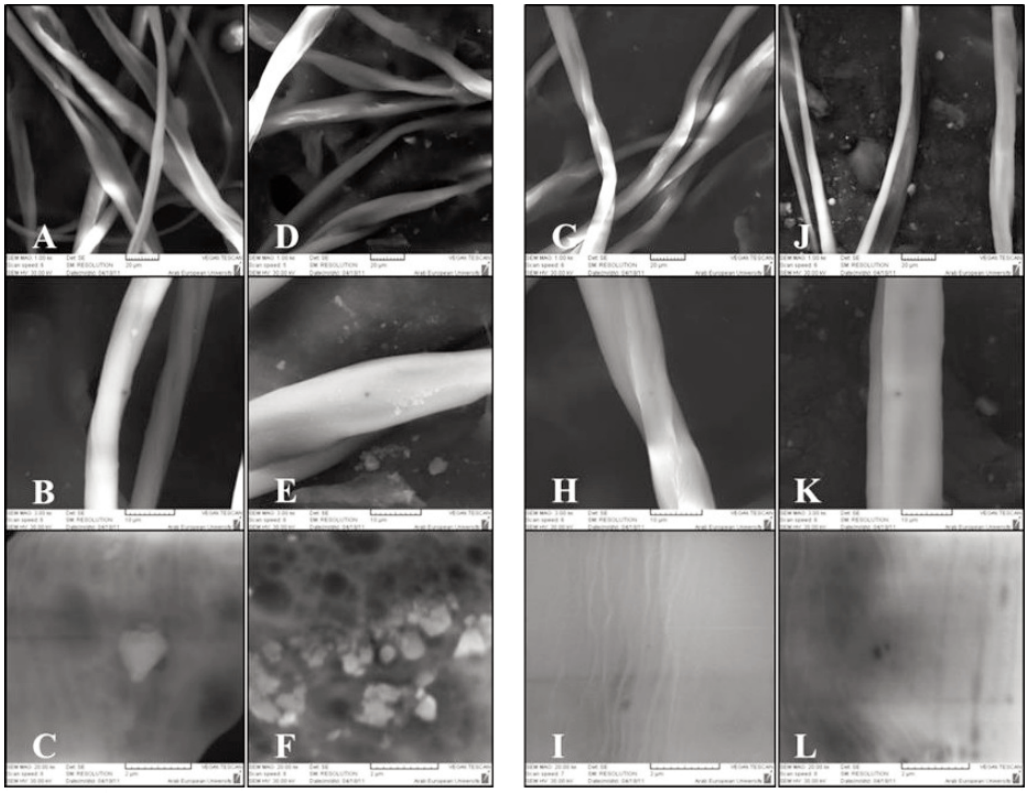



Fig. 7 - SEM images of cotton fibers. A, B and C. fibers of control 'Aleppo 118' under 1.00, 3.00 and 20.00 kx magnifications. D, E and F. fibers of treated 'Aleppo 118' under 1.00, 3.00 and 20.00 kx magnifications. G, H and I. fibers of control 'Deir Al Zour 22' under 1.00, 3.00 and 20.00 kx magnifications. J, K and L. fibers of treated 'Deir Al Zour 22' under 1.00, 3.00 and 20.00 kx magnifications.

trols. On the other hand, Aleppo 118 cultivar showed an almost steady expression pattern of the three genes in the two stages (high expression in S1 and low in S2). This continuous high expression activity of *DREB 1A* needs to be further investigated in 'Deir Al-Zour 22' to study its relation to water deficit tolerance.

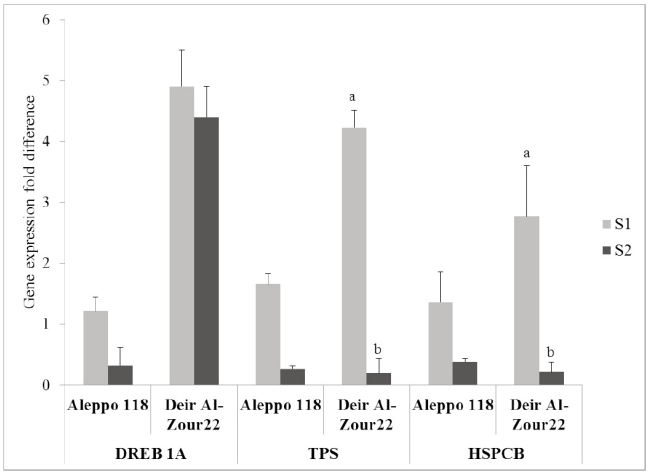



Fig. 8 - Fold expression of DREB1A, TPS and HSPCB genes in leaves of treated 'Aleppo 118' and 'Deir Al-Zour 22' plants. Leaf samples were collected in two stages, S1 (during floral bud to flower transition stage) and S2 (bolls opening and maturation). Data were subjected to Duncan's test with a confidence level of 95% using STATISTICA program. Significant fold expression change of TPS and HSPCB genes was observed between S1 and S2 treated 'Deir Al-Zour 22'. Data represent means and standard deviation of three replications.

## 4. Discussion and Conclusions

This study overlooks the behavior of two accredited cotton cultivars subjected to two irrigation regimes along their life cycle. It is anticipated that water scarcity in the Mediterranean region will increase under climatic change and this can be addressed by evaluating the impacts of climate change on water resources and their management, the adaptive capacity and the policy responses (Iglesias *et al.*, 2011).

Improving water resources management is challenged by the physiology and biology of the crop, irrigation practices, environment, farmers' perspectives, and government funding. Increasing crop water productivity, i.e. producing more food, income, better livelihoods and ecosystem services with less water (Molden *et al.*, 2010), has been a major interest to agronomists, farmers, environmentalists and economists.

Our research is mainly interested in understanding the responses of two credited Syrian cotton cultivars grown under water-shortage stress conditions, motivated by the concept of reaching a better quality and quantity of cotton fiber coupled with saving ground water resources in a cotton growing country.

Recently, drip irrigation systems have been widely employed, due to their improvement of water use efficiency (Patanè *et al.*, 2011). Hence, our field experiments were irrigated using the drip irrigation system to reduce water runoff losses.

Two credited cotton local cultivars, Aleppo 118 and Deir Al-Zour 22, have been investigated in our study. The two cultivars are assigned by the Ministry of Agriculture to be grown in their pertinent environments in the Syrian land. 'Deir Al-Zour 22' is the featured cultivar for cultivation in Deir Al-Zour Province, along the Euphrates, where high temperature is the dominant feature of the area especially during the growing season. 'Aleppo 118', an early maturity cultivar, is cultivated in the arid and semi-arid Northern-West regions of Syria, mostly in Aleppo province.

Control (full irrigation) and treated (deficit irrigation) plants of both cultivars were tested using some physiological, morphological, molecular, and biochemical parameters. The restricted water regime applied on our treated plants showed mild, non-significant effects on chlorophyll a and b content, which indicates a weak impact of the water-shortage stress regime on the photosynthesis process. However, the cultivar Aleppo 118 showed a slight reduction in chlorophyll a/b ratio in treated plants compared to a larger reduction in treated plants of 'Deir Al-Zour 22', which indicate the presence of higher chlorophyll b content in treated 'Deir Al-Zour 22' plants. This in turn may suggest a slower degradation process of chl b coming from a decrease in the enzymatic activities that are known to render the conversion of chl b to chl a (Folly and Engel, 1999). It is suggested that drought stress has to be prolonged and severe before the chloroplast start to break down (Jiang *et al.*, 2010). Another non-significant drop was also observed in the osmotic potential values of treated plants in both cultivars. However, the cultivar Aleppo 118 showed a greater osmotic adjustment value which may indicate its stronger turgor maintenance mechanism. Osmotic adjustment has been also associated with maintenance of membranes and protein structure, protection against oxidative damage (Dacosta and Huang, 2006). It has been reported that the greater the stress duration (increasing the number of stress cycles), the larger the osmotic adjust-

ment in cotton leaves (Oosterhuis and Wullschleger, 1987).

The morphology and phenology of plants can be significant indicators of drought tolerance. Changes in plant growth rate and/or in flowering time are two common strategies that plants use to cope with drought (Schmalenbach *et al.*, 2014).

The allotetraploid *G. hirsutum* L. species has a distinct growth manner in which the plant keeps a systematic morphological architecture (Khan, 2003) that can be useful in monitoring growth and developmental changes under diverse environmental conditions (Jawdat *et al.*, 2012). In general, physiological processes are induced in plants under stress conditions to reduce the cellular damage and to alter developmental timing to complete their life cycle fittingly (Yaish *et al.*, 2011). Stress-mediated flowering has been reported in several plant species; stresses like ultraviolet C, poor nutrition, low temperature and drought (Wada and Takeno, 2010). Our results noted earliness in treated plants of both varieties and a tendency of 'Aleppo 118' to bear higher percentage of flowers and floral buds toward the end of season. 'Aleppo 118' has been reported to show excessive vegetative growth under excess water and nitrogen compared to other local cultivars (CRA, personal communication). This may suggest that water status (excess and shortage) may hold back or trigger floral cues, and this needs to be investigated fully taking into consideration our result of the higher osmotic adjustment in 'Aleppo 118' compared to 'Deir Al-Zour 22'.

The small increase in stem height in treated plants of both cultivars can be explained that plants accumulate reserves in shoots and stems to cope with water stress (Chavez *et al.*, 2002). The water deficit regime, applied in our study through drip irrigation, encouraged the increase in number of secondary roots in treated plants of both cultivars on the expenses of a decrease in tap root length.

Fiber quality, which is a result of interactions between genetics and environment, controls fiber prices of cotton textile products (Wang *et al.*, 2014). The quest for cotton growers is the balance between fiber quality and quantity. Achieving such a balance in cotton cultivation regions with growing water shortages is a main concern to the agricultural and industrial sectors. An escalating number of publications aim at understanding the mechanism of cotton fiber development and adaptation to abiotic stresses, such as drought, for the improvement of cotton fiber yields and quality (Zhu *et al.*, 2011; Deeba *et al.*, 2012; Padmalatha *et al.*, 2012; Bowman *et al.*, 2013; Riaz *et al.*, 2013; Wang *et al.*, 2013; Zhang *et al.*, 2013; Sekmen *et al.*, 2014; Xie *et al.*, 2015). Cotton growth and yield are severely affected by excessive water-shortage stress, especially at critical growth stages such as the flowering phase (Dağdelen *et al.*, 2009). Soil water was found to be correlated with fiber strength, elongation (Johnson *et al.*, 2002) and fiber maturity (Davidonis *et al.*, 2004). Our results showed that two fiber properties, cohesion and micronaire, had no significant differences between control and treated plants of the two cultivars. It is with fiber cohesion, a property that causes materials to cling together, yarn spinning from staple fiber is possible (Wakelyn, 2007). A previous study showed fiber cohesion stability of 'Deir Al-Zour 22' between seasons and locations; whereas, 'Aleppo 118' showed cohesion stability between seasons in one location (Jawdat *et al.*, 2012). As for fiber micronaire, it reflects fiber fineness and is a measure of internal fiber thickness and deposits of cellulose. It has a desired value range in the international market between 3.5 and 4.9 (Jawdat *et al.*, 2012). Fiber micronaire, showed stability in both 'Aleppo 118' and 'Deir Al-Zour 22' between seasons and locations (Jawdat *et al.*, 2012). This indicates that environmental factors including water deficit have weak impact on the two fiber properties, cohesion and micronaire. Deficit irrigation showed no significant effect on fiber length and strength in Aleppo 118 cultivar. However, it had significant impact on the two fiber properties, causing increment in both strength and length of fiber in treated plants of Deir Al-Zour 22 cultivar. Fiber strength is an indicator of fiber resistance to stretching (Wang *et al.*, 2014) and is determined by environmental conditions and cultivar traits (Zhou *et al.*, 2011). It has been found that fiber strength is correlated with daily mean temperature during fiber development (Hanson and Ewing, 1956; Ma *et al.*, 2006; Wang *et al.*, 2009). This has been also observed on a previous study where a difference of 4-5°C showed a significant effect on fiber strength in both 'Aleppo 118' and 'Deir Al-Zour 22' (Jawdat *et al.*, 2012). However, this temperature difference did not affect fiber length in both cultivars (Jawdat *et al.*, 2012). This shows the impact of each of water deficit and temperature on fiber strength of 'Deir Al-Zour 22' compared to the impact of only temperature on fiber strength of 'Aleppo 118'. Fiber maturity can be defined as the relative wall thickness and wall development (Wakelyn, 2007). In our work, we observed an increase in fiber maturity factor values in treated

plants compared to the controls, which suggests that water deficit did not affect the maturity of cotton fiber in a negative way and hence their quality as related to dye-ability and ease of processing.

The FTIR-ATR spectra of the cotton fibers obtained from control and treated Aleppo 118 and Deir Al-Zour 22 cultivars showed similar spectra range except for few noticeable bands. This can be due to a reduction in surface water adsorption which leads to stronger fibers. The minimized accessibility of water molecules to the internal hydroxyl groups can be mostly due to the formation of cellulose macromolecules that induce a re-organization of cellulose and also increase the ordered cellulose (or crystalline) portion to produce a stronger fiber (Liu, 2013) in Deir Al-Zour 22 treated cultivar. Results of XRD showed minor CI variation between fibers from control and treated Deir Al-Zour 22. Both TGA and DSC showed no variation between fibers of treated and control plants in both cultivars.

Our work has also investigated gene fold change in expression of the transcription factor gene (*DREB1A*), the molecular chaperone gene (*HSPCB*) (Sotirios *et al.*, 2006), and the trehalose biosynthesis gene (*TPS*) (Kosmas *et al.*, 2006). The two genes *HSPCB* and *TPS* have been found in few studies to be expressed differently between drought-tolerant and drought-sensitive cotton genotypes. While the *HSPCB* showed differential expression during the drought period in leaves of drought tolerant genotypes and not in the sensitive ones (Nepomuceno *et al.*, 2002; Voloudakis *et al.*, 2002), the *TPS* gene was induced during the water stress period in both tolerant and sensitive cultivars (Nepomuceno *et al.*, 2002). Interestingly, our study showed a significant reduction in *HSPCB* and *TPS* genes activity in leaves during bolls opening and maturation in both cultivars. Whereas, *DREB 1A* gene kept a high active mode in leaves of treated 'Deir Al-Zour 22' plants compared to 'Aleppo 118' treated plants during that stage. Dehydration-responsive element binding proteins (DREBs) are transcription factors known to activate the expression of abiotic stress-responsive genes in divergent species via specific binding to the dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting element in promoters of target genes (Mizoi *et al.*, 2012). In *Arabidopsis*, the overexpression of *DREB1A* revealed both freezing and dehydration tolerance in transgenic plants (Liu *et al.*, 1998). A better drought and salt tolerance was observed in transgenic wheat plants with *DREB1* from *Glycine max* (Shiqing *et al.*, 2005). The overexpression of *Oryza sativa DREB1* in rice transgenic plants has also showed improved tolerance to drought, salt and low temperature stresses (Dubouzet *et al.*, 2003; Ito *et al.*, 2006). A group of researchers found that *DREB 1A* was induced at a high level in pistils, three days after drought treatment which suggests its possible role as an early drought response regulator in the *Arabidopsis* flower (Su *et al.*, 2013). Our study adds to such findings and points to *DREB 1A* constant up-regulation in leaves of 'Deir Al-Zour 22' under drought stress, during flowering and boll maturation.

Our study has presented the responses of two accredited cotton cultivars, Aleppo 118 and Deir Al-Zour 22 which have shown different behavior under water shortage. Both cultivars have shown early flowering. However, a larger percentage of treated 'Aleppo 118' plants kept flowering towards end of season compared to treated 'Deir Al-Zour 22' plants. This may indicate a potential survival mechanism and fruiting cycle extension in Aleppo 118 cultivar under water deficit regime, compared to the cultivar Deir Al-Zour 22.

Aleppo 118 cultivar displayed more consistent fiber quality between control and treated plants, while 'Deir Al-Zour 22' showed variation in fiber length and strength between control and treated plants. Both cultivars kept consistent micronaire and cohesion under normal and water deficit conditions. Two critical stages in cotton life cycle, flowering and boll development, were screened for *HSPCB*, *TPS* and *DREB 1A* gene activity using leaf material. Results have demonstrated an increase in fold expression of these genes in the flowering stage of treated plants compared to the controls. A large regression in genes activity was noticed during boll development except for *DREB1A* gene in treated 'Deir Al-Zour 22' plants. *DREB1A* gene showed continuous activation in the two stages and can be considered a candidate gene to support water deficit stress tolerance mechanism in this cultivar.

The question of which cultivar is more drought-tolerant than the other is to be deeply investigated in both cultivars, since each cultivar has shown different approach towards water-shortage stress tolerance.

**Acknowledgements**

The authors would like to thank the Director General of AECS and the Head of Molecular Biology and Biotechnology Department for their support. The

authors would like to extend their thanks to Mrs. Intissar Kara Joli for lab assistance, Dr. Moufak Roukaya for XRD analysis, the Physics department for SEM imaging and the agrarian office at the AECS for land and drip irrigation preparation.

**References**

ABIDI N., CABRALES L., HAIGLER C.H., 2014 - *Changes in the cell wall and cellulose content of developing cotton fibers investigated by FTIR spectroscopy*. - Carbohydr. Polym., 100(0): 9-16.

ARTICO S., NARDELI S.M., BRILHANTE O., GROSSI-DE-SA M.F., ALVES-FERREIRA M., 2010 - *Identification and evaluation of new reference genes in* Gossypium hirsutum *for accurate normalization of real-time quantitative RT-PCR data*. - BMC Plant Biol., 10: 49.

BOWMAN M.J., PARK W., BAUER P.J., UDALL J.A., PAGE J.T., RANEY J., SCHEFFLER B.E., JONES D.C., CAMPBELL B.T., 2013 - *RNA-Seq transcriptome profiling of upland cotton (*Gossypium hirsutum *L.) root tissue under water-deficit stress*. - PLoS One, 8 (12): e82634.

CHA-UM S., SUPAIBULWATANA K., KIRDMANEE C., 2006 - *Water relation, photosynthetic ability and growth of Thai Jasmine rice (*Oryza sativa *L. ssp. indica cv. KDML 105) to salt stress by application of exogenous glycine-betaine and choline*. - J. Agro. Crop Sci., 192(1): 25-36.

CHAVEZ M.M., PEREIRA J.S., MAROCO J., RODRIGUES M.L., RICARDO C.P.P., OSORIO M.L., CARVALHO I., FARIA T., PINHEIRO C., 2002 - *How plants cope with water stress. Photosynthesis and growth*. - Ann. Bot., 89: 907-916.

DACOSTA M., HUANG B., 2006 - *Osmotic adjustment associated with variation in bentgrass tolerance to drought stress*. - J. Amer. Soc. Hort. Sci., 131 (3): 338-344.

DAĞDELEN N., BAŞAL H., YILMAZ E., GÜRBÜZ T., AKÇAY S., 2009 - *Different drip irrigation regimes affect cotton yield, water use efficiency and fiber quality in western Turkey*. - Agric. Water Manage., 96(1): 111-120.

DAVIDONIS G.H., JOHNSON A.S., LANDIVAR J.A., FERNANDEZ C.J., 2004 - *Cotton fiber quality is related to boll location and planting date*. - Agron. J., 96(1): 42-47.

DEEBA F., PANDEY A.K., RANJAN S., MISHRA A., SINGH R., SHARMA Y.K., SHIRKE P.A., PANDEY V., 2012 - *Physiological and proteomic responses of cotton (*Gossypium herbaceum *L.) to drought stress*. - Plant Physiol. Biochem., 53(0): 6-18.

DUBOUZET J.G., SAKUMA Y., ITO Y., KASUGA M., DUBOUZET E.G., MIURA S., SEKI M., SHINOZAKI K., YAMAGUCHI-SHINOZAKI K., 2003 - *OsDREB genes in rice,* Oryza sativa *L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression*. - Plant J., 33(4): 751-763.

FERNIE A.R., SCHAUER N., 2009 - *Metabolomics-assisted breeding: a viable option for crop improvement?* - Trends Genet., 25(1): 39-48.

FOLLY P., ENGEL N., 1999 - *Chlorophyll b to chlorophyll a conversion precedes chlorophyll degradation in* Hordeum vulgare *L.* - J. Biol. Chem., 274(31): 21811-21816.

FUKAI S., COOPER M., 1995 - *Development of drought-resistant cultivars using physiomorphological traits in rice*. - Field Crops Res., 40(2): 67-86.

HANSON R.G., EWING E.C., 1956 - *Effect of environmental factors on fiber properties and yield of deltapine cottons*. - Agron. J., 48 (12): 573-581.

IGLESIAS A., GARROTE L., DIZ A., SCHLICKENRIEDER J., MARTIN-CARRASCO F., 2011 - *Re-thinking water policy priorities in the Mediterranean region in view of climate change*. - Environ. Sci. Policy, 14(7): 744-757.

ITO Y., KATSURA K., MARUYAMA K., TAJI T., KOBAYASHI M., SEKI M., SHINOZAKI K., YAMAGUCHI-SHINOZAKI K., 2006 - *Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice*. - Plant Cell Physiol., 47(1): 141-153.

JAWDAT D., HILALI M.A., AYYOUBI Z., ELIAS R., AL-RAYAN R., AL-SALTI M.N., AL-SAFADI B., 2012 - *Response of cotton varieties to different environments: flowering behavior and fiber quality*. - Pak. J. Agri. Sci., 49(3): 289-298.

JAWDAT D., KARAJOLI I., 2012 - *A modified and inexpensive protocol for the rapid isolation of RNA from diverse plant species*. - J. Hortic. Sci., 87(4): 317-324.

JIANG Y., WATKINS E., LIU S., YU X., LUO N., 2010 - *Antioxidative responses and candidate gene expression in prairie junegrass under drought stress*. - J. Amer. Soc. Hort. Sci., 135(4): 303-309.

JOHNSON R.M., DOWNER R.G., BRADOW J.M., BAUER P.J., SADLER E.J., 2002 - *Variability in cotton fiber yield, fiber quality, and soil properties in a southeastern coastal plain*. - Agron. J., 94(6): 1305-1316.

KHAN H.R., PAULL J.G., SIDDIQUE K.H.M., STODDARD F.L., 2010 - *Faba bean breeding for drought-affected environments: a physiological and agronomic perspective*. - Field Crops Res., 115 (3): 279-286.

KHAN U.Q., 2003 - *Monitoring the growth and development of cotton plants using main stem node counts*. - Asian J. Plant Sci., 2(8): 593-596.

KOSMAS S., ARGYROKASTRITIS A., LOUKAS M., ELIOPOULOS E., TSAKAS S., KALTSIKES P., 2006 - *Isolation and characterization of drought-related trehalose 6-phosphate-synthase gene from cultivated cotton (*Gossypium hirsutum *L.)*. - Planta, 223(2): 329-339.

LIU Q., KASUGA M., SAKUMA Y., ABE H., MIURA S., YAMAGUCHI-SHINOZAKI K., SHINOZAKI K., 1998 - *Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in* Arabidopsis. - Plant Cell, 10(8): 1391-1406.

LIU Y., 2013 - *Recent progress in fourier transform infrared (FTIR) spectroscopy study of compositional, structural*

*and physical attributes of developmental cotton fibers*. - Materials, 6(1): 299.

MA F., ZHU Y., CAO W., YANG J., ZHENG Z., CHENG H., MU C., 2006 - *Modeling fiber quality formation in cotton*. - Zuo wu xue bao, 32(3): 442-448.

MEYRE D., LEONARDI A., BRISSON G., VARTANIAN N., 2001 - *Drought-adaptive mechanisms involved in the escape/tolerance strategies of* Arabidopsis Landsberg erecta *and Columbia ecotypes and their F1 reciprocal progeny*. - J. Plant Physiol., 158(9): 1145-1152.

MIZOI J., SHINOZAKI K., YAMAGUCHI-SHINOZAKI K., 2012 - *AP2/ERF family transcription factors in plant abiotic stress responses*. - Biochim. Biophys. Acta, 1819(2): 86-96.

MOLDEN D., OWEIS T., STEDUTO P., BINDRABAN P., HANJRA M.A., KIJNE J., 2010 - *Improving agricultural water productivity: Between optimism and caution*. - Agric. Water Manage., 97(4): 528-535.

NEPOMUCENO A.L., OOSTERHUIS D., STEWART J.M., TURLEY R., NEUMAIER N., FARIAS J.R.B., 2002 - *Expression of heat shock protein and trehalose-6-phosphate synthase homologues induced during water deficit in cotton*. - Braz. J. Plant Physiol., 14: 11-20.

OOSTERHUIS D.M., WULLSCHLEGER S.D., 1987 - *Osmotic adjustment in cotton (*Gossypium hirsutum *L.) leaves and roots in response to water stress*. - Plant Physiol., 84(4): 1154-1157.

OWEIS T.Y., FARAHANI H.J., HACHUM A.Y., 2011 - *Evapotranspiration and water use of full and deficit irrigated cotton in the Mediterranean environment in northern Syria*. - Agric. Water Manage., 98(8): 1239-1248.

PADMALATHA K.V., DHANDAPANI G., KANAKACHARI M., KUMAR S., DASS A., PATIL D.P., RAJAMANI V., KUMAR K., PATHAK R., RAWAT B., LEELAVATHI S., REDDY P.S., JAIN N., POWAR K.N., HIREMATH V., KATAGERI I.S., REDDY M.K., SOLANKE A.U., REDDY V.S., KUMAR P.A., 2012 - *Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defense responsive genes*. - Plant Mol. Biol., 78(3): 223-246.

PATANÈ C., TRINGALI S., SORTINO O., 2011 - *Effects of deficit irrigation on biomass, yield, water productivity and fruit quality of processing tomato under semi-arid Mediterranean climate conditions*. - Sci. Hort., 129(4): 590-596.

REDDY A.R., CHAITANYA K.V., VIVEKANANDAN M., 2004 - *Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants*. - J. Plant Physiol., 161(11): 1189-1202.

RIAZ M., FAROOQ J., SAKHAWAT G., MAHMOOD A., SADIQ M.A., YASEEN M., 2013 - *Genotypic variability for root/shoot parameters under water stress in some advanced lines of cotton (*Gossypium hirsutum *L.)*. - Genet. Mol. Res., 12(1): 552-561.

SCHMALENBACH I., ZHANG L., REYMOND M., JIMENEZ-GOMEZ J.M., 2014 - *The relationship between flowering time and growth responses to drought in the* Arabidopsis Landsberg erecta *x Antwerp-1 population*. - Front. Plant Sci., 5

SEKMEN A.H., OZGUR R., UZILDAY B., TURKAN I., 2014 - *Reactive oxygen species scavenging capacities of cotton (*Gossypium hirsutum*) cultivars under combined drought and heat induced oxidative stress*. - Environ. Exp. Botany, 99(0): 141-149.

SHIQING G., HUIJUN X., XIANGUO C., MING C., ZHAOSHI X., LIANCHENG L., XINGGUO Y., LIPU D., XIAOYAN H., YOUZHI M., 2005 - *Improvement of wheat drought and salt tolerance by expression of a stress-inducible transcription factorGmDREB of soybean (Glycine max)*. - Chin. Sci. Bull., 50(23): 2714-2723.

SOTIRIOS K., ARGYROKASTRITIS A., LOUKAS M., ELIOPOULOS E., TSAKAS S., KALTSIKES P., 2006 - *Isolation and characterization of stress related Heat shock protein calmodulin bindinggene from cultivated cotton (*Gossypium hirsutum *L.)*. - Euphytica, 147(3): 343-351.

SU Z., MA X., GUO H., SUKIRAN N.L., GUO B., ASSMANN S.M., MA H., 2013 - *Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in* Arabidopsis. - Plant Cell, 25(10): 3785-3807.

TUBEROSA R., SALVI S., 2006 - *Genomics-based approaches to improve drought tolerance of crops*. - Trends Plant Sci., 11(8): 405-412.

ÜNLÜ M., KANBER R., KOÇ D.L., TEKIN S., KAPUR B., 2011 - *Effects of deficit irrigation on the yield and yield components of drip irrigated cotton in a mediterranean environment*. - Agric. Water Manage., 98(4): 597-605.

VOLOUDAKIS A.E., KOSMAS S.A., TSAKAS S., ELIOPOULOS E., LOUKAS M., KOSMIDOU K., 2002 - *Expression of selected drought-related genes and physiological response of Greek cotton varieties*. - Funct. Plant Biol., 29(10): 1237-1245.

WADA K.C., TAKENO K., 2010 - *Stress-induced flowering*. - Plant Signal Behav, 5(8): 944-947.

WAKELYIN P.H., 2006 - *Cotton fiber chemistry and technology*. - CRC Press, Boca Raton, FL, USA, pp. 123.

WANG M., WANG Q., ZHANG B., 2013 - *Response of miRNAs and their targets to salt and drought stresses in cotton (*Gossypium hirsutum *L.)*. - Gene, 530(1): 26-32.

WANG X., ZHANG L., EVERS J.B., MAO L., WEI S., PAN X., ZHAO X., VAN DER WERF W., LI Z., 2014 - *Predicting the effects of environment and management on cotton fibre growth and quality: a functional-structural plant modelling approach*. - AoB Plants, 6 (0): plu040-.

WANG Y., SHU H., CHEN B., McGIFFEN M. Jr., ZHANG W., XU N., ZHOU Z., 2009 - *The rate of cellulose increase is highly related to cotton fibre strength and is significantly determined by its genetic background and boll period temperature*. - Plant Growth Regul., 57(3): 203-209.

XIE F., WANG Q., SUN R., ZHANG B., 2015 - *Deep sequenc-*

*ing reveals important roles of microRNAs in response to drought and salinity stress in cotton*. - J. Exp. Bot., 66(3): 789-804.

YAISH M.W., COLASANTI J., ROTHSTEIN S.J., 2011 - *The role of epigenetic processes in controlling flowering time in plants exposed to stress*. - J. Exp. Bot., 62(11): 3727-3735.

ZHANG J., LI D., ZOU D., LUO F., WANG X., ZHENG Y., LI X., 2013 - *A cotton gene encoding a plasma membrane aquaporin is involved in seedling development and in response to drought stress*. - Acta Biochim. Biophys. Sin., 45(2): 104-114.

ZHOU Z., MENG Y., WANG Y., CHEN B., ZHAO X., OOSTERHUIS D.M., SHU H., 2011 - *Effect of planting date and boll position on fiber strength of cotton (*Gossypium hirsutum *L.)*. - Am. J. Exp. Agri., 1(4): 331-342.

ZHU L.-F., HE X., YUAN D.-J., XU L., XU L., TU L.-L., SHEN G.-X., ZHANG H., ZHANG X.-L., 2011 - *Genome-wide identification of genes responsive to ABA and cold/salt stresses in* Gossypium hirsutum *by data-mining and expression pattern analysis*. - Agri. Sci. China, 10(4): 499-508.

Adv. Hort. Sci., 2018 32(1): 93-103 DOI: 10.13128/ahs-21874

AHS
Advances in Horticultural Science

# Physio-morphological variations of pummelo genotype (*Citrus grandis* L. Osbeck)

**M.M. Hossain*, R.F. Disha, M.A. Rahim**
*Department of Horticulture, Bangladesh Agricultural University, Mymensingh, Bangladesh.*

*Key words*: *Citrus grandis* L. Osbeck, correlation coefficient, heritability, path analysis.



(*) **Corresponding author:**
mokter.agr@bau.edu.bd

**Citation:**
HOSSAIN M.M., DISHA R.F., RAHIM M.A., 2018 - *Physio-morphological variations of pummelo genotype (Citrus grandis L. Osbeck).* - Adv. Hort. Sci., 32(1): 93-103



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 10 October 2017
Accepted for publication 12 January 2018

**Abstract: The study was conducted to evaluate the physio-morphological variations of 21 pummelo genotype. The experiment was carried out at the existing plantation of Bangladesh Agricultural University Germplasm Centre, Mymensingh, during September 2014 to June 2015. Results showed that different genotype exhibited differently in their physio-morphological features. Genotype Thai Jambura exhibited highest leaf and petiole wing length (16.77 cm and 11.63 cm, respectively), while maximum number of anthers (44.33) were recorded in genotype Green skin. The heaviest and lightest fruits were recorded in genotype Hybrid (1283.33 g) and Accession-52 (300 g). While the maximum weight of non-edible portion (463.33 g), pulp to peel ratio (3.97), thickness of pulp (11.50 cm), amount of juice (366.67 ml), total soluble solids (TSS) (18.67%), number of seeds (114) and weight of seeds (58 g) were found in genotype Hybrid. Correlation coefficient study indicated that leaf length, breadth, petiole wing length, fruit weight, weight of non-edible portion, seed weight, seed number/fruit had positive and highly significant association with leaf breadth, petiole wing breadth, weight of non-edible portion, pulp thickness, total weight of seeds/fruit and number of fruits/plant, respectively. In respect of path analysis, leaf breadth, petiole wing length, fruit weight, average weight of seed, %TSS, seed number/fruit had positive direct effect on fruits/plant indicating its importance as a selection criteria.**

## 1. Introduction

Pummelo (*Citrus grandis* L. Osbeck) belonging to the family Rutaceae, is one of the most distinctive and easily recognized species of the genus citrus (Verdi, 1988). It is one of the most important fruit in Bangladesh because of its taste, aroma and nutrient value. Citrus carry some vitamins like vitamins C, B, A and some minerals like calcium, iron, citric acid etc. The pummelo is an exotic large citrus fruit that is an ancestor of the common grapefruit. In Bangladesh it is locally known as jambura, batabilabu,

badami, jamir. The pummelo is significantly larger than the grapefruit. Its flesh is sweet and it has a thick skin and rind. The fruit of the pummelo has a light green colored rind but this gradually becomes mostly yellow when it has fully ripened. The inside of the fruit has a pink color when it is ripe. The pummelo tree thrives well in tropical or near tropical climates. Like other citrus fruits, the pummelo usually ripen in winter. It is an important commercial citrus fruit, grown and available almost everywhere in Bangladesh. The availability of this fruits helping people to overcome the malnutrition problem. There is a special pummelo fruit based diet to treat asthma. Pummelo is a dietary fruit; its caloric value is 25-58 kcal/100 g (Morton, 1987). In Vietnam, the aromatic flowers are used in making perfume. The wood is used for tool handles and firewood while leaves, flowers, fruits, and seeds are sometimes used as herbal medicine to treat cough, fever and gastric disorders (Verheij and Coronel, 1992).

Pummelo grows well everywhere in Bangladesh and is comparatively more tolerate to insects and diseases than other citrus fruits. But the number of trees with good quality fruit is very negligible in comparison to other citrus growing countries of the world. In the year 2015, Bangladesh produces 31,036 metric tons pummelo from 4159.10 hectares of land (BBS, 2015). Although it is one of the most important citrus fruit in Bangladesh, production as well as yield of pummelo fruits is very low due to lack of high yielding and good quality variety. It is reported that a single pummel tree can yield 70-100 fruits/year which is equivalent to 20 tons/ha/year (Verheij and Coronel, 1992). Therefore, selection of high yielding genotype is necessarily important to increase the yield of pummelo in Bangladesh.

Characterization of pummelo using morphological traits will help in the selection of genetically potential genotype for cultivation and also for their exploitation in plant breeding program. Selection of superior genotype from the collected accessions will help in increasing production of pummelo in this country. For improving the production as well as yield of pummelo fruits in this country, Bangladesh Agriculture University - Germplasm Center (BAU-GPC) has already been collected some pummelo genotype from different corner of Bangladesh and also from Thailand, Vietnam, and Malaysia. This experiment has been undertaken to study the physio-morphological characteristics of those pummel accessions to evaluate their relative performance. In plant breeding program, knowledge of the interrelationship among and between yield contributing characters is necessary. Correlation and the path coefficient analysis will provide a true picture of genetic associating among different traits (Bhatt, 1973). Path coefficient analysis specifics the cause and effect and measures their relative importance. Therefore, correlations in combination with the path co-efficient analysis quantify the direct and indirect contribution of one character upon another (Dewey and Lu, 1959). This experiment was therefore, undertaken to study the physio-morphological characteristics of pummelo genotype collected from different parts of the world and to access the interrelationship between yield and yield contributing characters and to select better quality genotype for cultivation in Bangladesh.

## 2. Materials and Methods

The experiment was conducted at Bangladesh Agricultural University Germplasm Center, Department of Horticulture, Bangladesh Agricultural University, Mymensingh during September 2014 to June 2015. The location of BAU-GPC was medium high land, well drained and slightly acidic soil with pH range from 5.5 to 6.8. The study was conducted in an established orchard where 21 indigenous and exotic pummelo genotype were collected and established (Table 1). The average age of plants were 3-4 years.

Table 1 - Collected pummelo genotype with their source of origin

| Treatment | Pummelo genotype | Sources of origin |
|---|---|---|
| $T_1$ | BAU-1 | Bangladesh |
| $T_2$ | BAU-2 | ,, |
| $T_3$ | BAU-4 | ,, |
| $T_4$ | Hybrid | ,, |
| $T_5$ | Jambura (seeded) | ,, |
| $T_6$ | Mohini | ,, |
| $T_7$ | Green skin | Vietnam |
| $T_8$ | Malaysian | Malaysia |
| $T_9$ | Thai jambura | Thailand |
| $T_{10}$ | Accession-51 | Bangladesh |
| $T_{11}$ | Accession-52 | ,, |
| $T_{12}$ | Accession-57 | Bangladesh |
| $T_{13}$ | Accession-58 | ,, |
| $T_{14}$ | Accession-59 | ,, |
| $T_{15}$ | Accession-62 | ,, |
| $T_{16}$ | Accession-63 | ,, |
| $T_{17}$ | Accession-76 | ,, |
| $T_{18}$ | Accession-87 | ,, |
| $T_{19}$ | Accession-93 | ,, |
| $T_{20}$ | Accession-101 | ,, |
| $T_{21}$ | Accession-103 | ,, |

These 21 genotype were considered as experimental treatments. The experiment was carried out in a randomized complete block design with three replications. Data on different physio-morphological characters of leaves, flowers, fruits and seeds were recorded from sample plants of each genotype following citrus IPGRI descriptor (IPGRI, 1999). Leaf length, breadth, petiole wing length, petiole wing breadth were measured by using measuring scale. Individual fruit weight was recorded by a digital balance and expressed in grams (g). For determination of pulp to peel ratio, fruits were peeled off and weight was taken for separated pulp and peel and expressed in g. Thickness of fruit pulp and peel was measured by using measuring tape after cross-sectioning of the fruits and expressed in centimeter (cm). The pulp vesicles were removed and blended in a blenderin addition with 150 ml of water. Then juice was filtered by a sieve. Except juice the rest of waste materials was considered as non-edible portion and recorded the weight. The amount of juice was measured by measuring cylinder and expressed in milliliter (ml). Total soluble solids (TSS) was determined by Abbe Hand Refractometer. A drop of juice squeezed from the pulp vesicles of fruit then placed on the prism of the refractometer and percent total soluble solids was observed from reading. Temperature correction was made using the method describe by Ranganna (1978).Total number of fruits of each plant was counted during 15 days interval. Thereafter, the average number of fruits per plant was recorded. The total number of seeds per fruit was counted. The total number of seeds, which was obtained from fruits, was weighted in a balance and expressed in g. After counting and weighing the total seeds average weight of seed was determined by the following formula and expressed in g.

$$\text{Average weight of seed (g)} = \frac{\text{weight of seeds (g)}}{\text{total number of seeds}}$$

*Estimation of simple correlation coefficient*

Association of different characters under the study was analyzed by working out simple correlation coefficient for all the possible pairs of character combination. Simple correlation coefficient (r) among the important characters of pummelo genotype was estimated with the formula stated by Singh and Chaudhary (1985).

*Estimation of path coefficient*

Path coefficient analysis was done according to the procedure stated by Dewey and Lu (1959) using simple correlation values. In path analysis, correlation coefficient is partitioned into direct and indirect effects of independent variable on the dependent variable. In order to estimate direct and indirect effects of the correlated characters, i.e.$X_1$, $X_2$ and $X_3$, yield Y, a set of simultaneous equations (three equations in the examples) is required to be formulated as shown below:

$$ryx_1 = Pyx_1 + Pyx_2rx_1x_2 + Pyx_3rx_1x_3, ryx_2 = Pyx_1rx_1x_2 + Pyx_2 + Pyx_3, ryx_3 = Pyx_1rx_1x_3 + Pyx_2rx_2x_3 + Pyx_3$$

Where, 'r' denotes simple correlation coefficient and 'P' denotes path coefficient.

Total correlation, say between $x_1$ and Y is thus partitioned as followed:

Pyx1 = indirect effect of x1, Pyx2rx1x3 = indirect effect of x1 via x2 only, Pyx3rx1x3 = indirect effect of x1 via x3 only.

After calculating the direct and indirect effect of the characters, residual effect (R) was calculated by using the formula stated by Singh and Chaudhary (1985).

*Component of variance*

The genotypic and phenotypic variances were calculated according to Johnson *et al*. (1955). Estimation of genotypic and phenotypic coefficient of variation were calculated according to Burton (1952).

*Estimation of heritability*

Heritability in broad sense can be calculated by using following formula:

$$H^2_b\,(\%) = \frac{\text{Genotipic variance}}{\text{Penotypic variance}} \times 100$$

*Statistical analysis*

Data on different physio-morphological parameters were statistically analyzed to find out the significance of difference among genotype means. The analyses of variances for most of the characters under consideration were performed by *F* variance test. The significance of the difference between treatments means was evaluated by least significance difference test describe by Gomez and Gomez (1984).

## 3. Results

*Morphological features of leaves*

As regard to leaf length it was observed that leaf length varied significantly among the genotype. The longest value was observed in genotype Thai Jambura (16.77 cm) and minimum value in genotype

BAU-2 (7.67 cm) (Fig. 1). It was revealed that the leaf breadth of the plant also varied significantly and ranged from 5.00 to 11.63 cm (Table 2). The genotype Thai Jambura showed the highest leaf breadth (11.63 cm) and the lowest leaf breadth (5 cm) was observed in Accession-62 (Table 2). It was revealed that the petiole wing length of the leaf varied significantly and ranged from 1.23 to 3.60 cm (Table 2). The genotype Thai Jambura showed the highest petiole wing length (3.60 cm) and the lowest petiole wing length (1.23 cm) was observed in Accession-62 (Table 2). Petiole wing breadth of pummelo genotype varied significantly. The genotype Thai Jambura showed the highest petiole wing breadth (4.17 cm) and the lowest petiole wing breadth (0.90 cm) was observed in Accession-63 (Table 2).

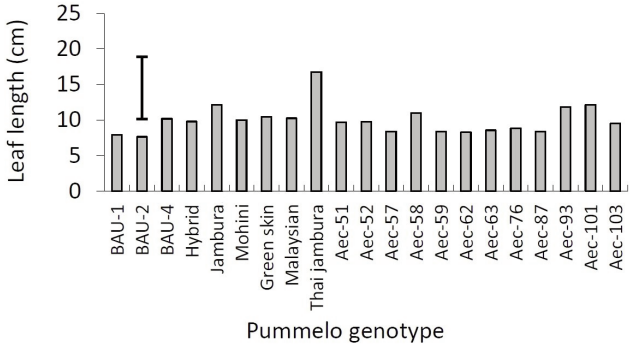



Fig. 1 - Leaf length of 21 pummelo genotype. Vertical bar indicates LSD at 1% level of probability.

*Morphological features of flowers*

In case of number of petals per flower it was observed that petals varied significantly genotype to genotype. The maximum number of petals was found in genotype Hybrid (6) and minimum value (4) in genotype BAU-2, BAU-4, Mohini, Accession-59, 63, 93 and 103 (Table 2). Similarly, calyx number was also found significantly varied among the genotype. The height number of calyx was observed in genotype Hybrid (6) and lowest number (4) in genotype BAU-2, BAU-4, Mohini, Accession-51, 59, 63, 93 and 103 (Table 2). However, number of anthers/flower significantly varied among 21 pummelo genotype and ranged from 22.67 to 44.33. The maximum value was recorded in genotype Green skin (44.33) and the minimum value in Accession-76 (22.67) (Table 2).

*Physio-morphological features of fruits*

Individual fruit weight varied significantly among the genotype and it ranged from 300 to 1283.33 g. Fruit with the heaviest weight (1283.33 g) was observed in genotype Hybrid and the lowest (300 g)

Table 2 - Leaf breadth, petiole wing length, petiole wing breadth, number of petals, calyx, anthers per flowers of 21 pummelo genotype

| Genotype | Leaf breadth (cm) | Petiole wing length (cm) | Petiole wing breadth (cm) | No. of petals/flower | No. of calyx/flower | No. of anthers/flower |
|---|---|---|---|---|---|---|
| BAU-1 | 5.70 | 2.00 | 1.60 | 5.00 | 5.00 | 42.00 |
| BAU-2 | 5.83 | 2.00 | 1.50 | 4.00 | 4.00 | 35.67 |
| BAU-4 | 5.93 | 2.83 | 2.87 | 4.00 | 4.00 | 39.00 |
| Hybrid | 5.93 | 1.67 | 0.93 | 6.00 | 6.00 | 32.33 |
| Jambura (Seeded) | 6.23 | 3.33 | 2.33 | 5.00 | 5.00 | 32.33 |
| Mohini | 6.17 | 2.17 | 1.67 | 4.00 | 4.00 | 38.00 |
| Green skin | 5.93 | 2.50 | 1.73 | 5.00 | 5.00 | 44.33 |
| Malaysian | 5.63 | 1.73 | 1.17 | 5.00 | 5.00 | 43.00 |
| Thai jambura | 11.63 | 3.6 | 4.17 | 5.00 | 5.00 | 41.33 |
| Accession-51 | 5.90 | 1.33 | 1.17 | 4.00 | 4.00 | 34.33 |
| Accession-52 | 5.17 | 2.70 | 2.23 | 5.00 | 5.00 | 42.67 |
| Accession-57 | 5.80 | 2.40 | 1.40 | 5.00 | 5.00 | 32.33 |
| Accession-58 | 8.17 | 1.67 | 1.50 | 5.00 | 5.00 | 41.00 |
| Accession-59 | 5.80 | 2.40 | 1.40 | 4.00 | 4.00 | 43.67 |
| Accession-62 | 5.00 | 1.23 | 1.10 | 5.00 | 5.00 | 31.33 |
| Accession-63 | 5.63 | 1.40 | 0.90 | 4.00 | 4.00 | 33.33 |
| Accession-76 | 5.17 | 2.40 | 2.50 | 5.00 | 5.00 | 22.67 |
| Accession-87 | 6.27 | 2.73 | 3.33 | 5.00 | 5.00 | 43.67 |
| Accession-93 | 7.13 | 2.10 | 2.00 | 4.00 | 4.00 | 35.33 |
| Accession-101 | 7.07 | 2.00 | 1.47 | 5.00 | 5.00 | 37.67 |
| Accession-103 | 5.43 | 1.83 | 1.33 | 4.00 | 4.00 | 29.00 |
| $LSD_{0.01}$ | 0.69 | 0.62 | 0.76 | 0.78 | 0.78 | 6.07 |
| Level of significance | ** | ** | ** | ** | ** | ** |

** = Significant at 1% level of probability.

fruit was recorded in Accession-52 (Table 3). In respect of non-edible portion weight, statistically significant variations were noticed among the genotype. The maximum weight of non-edible portion (463.33 g) was obtained from the genotype Hybrid. On the other hand, the minimum weight of non-edible portion (100 g) was recorded from the genotype Accession-52 (Table 3). Pulp to peel ratio of fruits varied significantly among the 21 pummelo genotype. The amount of pulp to peel ratio was highest in genotype Hybrid and lowest in Accession-87 (Fig. 2).

Among the genotype thickness of fruit pulp differed significantly and ranged from 6.50 to 11.50 cm (Table 3). The pulp of the fruits of genotype BAU-1 and BAU-2 was the thickest (11.50 cm) followed by the Hybrid genotype (11.37 cm) and Thai Jambura (11.00 cm) and the thinnest (6.33 cm) pulp was observed in Accession-51 (Table 3). The thickness of peel also differed significantly among the genotype. The peel of the genotype Jambura (seeded) and Accession-93 was thickest (2.17 cm) and the thinnest (1.00 cm) peel was observed in genotype Accession-76 and Accession-103 (Table 3). In terms of juice content of fruits it was noticed that pummelo genotype varied significantly. The highest amount of juice (366.67 ml) was measured in genotype BAU-1 and Hybrid followed by BAU-2 (350 ml) and Thai Jambura (336.67 ml) while the lowest (90 ml) found in Accession-52 (Table 3).

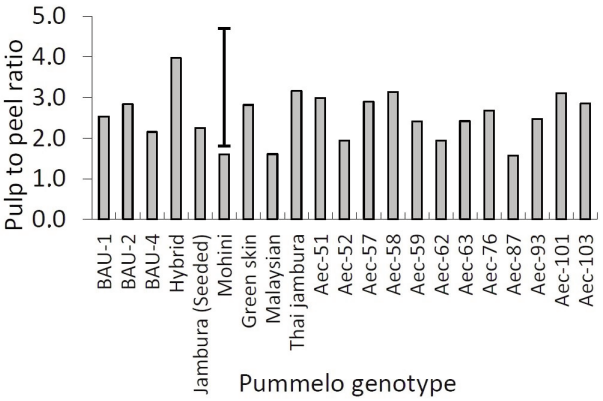



Fig. 2 - Pulp to peel ratio of 21 pummelo genotype. Vertical bar indicates LSD at 1% level of probability.

Total soluble solids (TSS) contents of fruits was significantly different in different pummelo genotype. The highest TSS was recorded in genotype Hybrid (18.67% Brix) followed by Accession-62 (18.23% Brix), Accession-63 and Accession-87 (17.33% Brix) and the lowest TSS was observed in genotype Mohini (13.5% Brix) (Fig. 3).

Table 3 - Fruit weight, weight of non-edible portion, thickness of pulp, peel, average weight of seed/ fruit, amount of juice/fruit and number of fruits/plant of 21 pummelo genotype

| Genotype | Fruit weight (g) | Non-edible portion weight (g/fruit) | Pulp thickness (cm) | Peel thickness (cm) | Juice amount (ml/fruit) | Number of fruits/plant | Seed weight average (g) |
|---|---|---|---|---|---|---|---|
| BAU-1 | 1160.00 | 450.00 | 11.50 | 2.03 | 366.67 | 20.16 | 0.50 |
| BAU-2 | 1250.00 | 426.67 | 11.50 | 1.83 | 350.00 | 17.38 | 0.48 |
| BAU-4 | 1060.00 | 266.67 | 10.17 | 1.83 | 143.33 | 33.33 | 0.47 |
| Hybrid | 1283.33 | 463.33 | 11.37 | 1.80 | 366.67 | 29.56 | 0.51 |
| Jambura (Seeded) | 696.67 | 290.00 | 8.97 | 2.17 | 233.33 | 47.16 | 0.24 |
| Mohini | 543.33 | 206.67 | 7.33 | 1.57 | 101.67 | 35.00 | 0.08 |
| Green skin | 866.67 | 243.33 | 10.50 | 1.30 | 223.33 | 10.00 | 0.33 |
| Malaysian | 1020.00 | 290.00 | 10.83 | 1.67 | 206.67 | 25.86 | 0.43 |
| Thai jambura | 1013.33 | 346.67 | 11.00 | 1.50 | 336.67 | 31.16 | 0.29 |
| Accession-51 | 516. 67 | 233.33 | 6.33 | 1.83 | 130.00 | 14.16 | 0.55 |
| Accession-52 | 300.00 | 100.00 | 6.50 | 1.33 | 90.00 | 14.53 | 0.58 |
| Accession-57 | 906.67 | 380.00 | 9.50 | 2.00 | 200.00 | 15.60 | 0.39 |
| Accession-58 | 490.00 | 236.67 | 7.33 | 1.33 | 140.00 | 11.36 | 0.06 |
| Accession-59 | 696.67 | 276.67 | 8.67 | 1.50 | 206.67 | 13.53 | 0.65 |
| Accession-62 | 613.33 | 306.67 | 8.17 | 2.00 | 123.33 | 9.20 | 0.42 |
| Accession-63 | 760.00 | 323.33 | 9.33 | 1.83 | 233.33 | 10.58 | 0.47 |
| Accession-76 | 640.00 | 136.67 | 9.73 | 1.00 | 193.33 | 12.24 | 0.45 |
| Accession-87 | 720.00 | 276.67 | 8.50 | 1.17 | 170.00 | 12.73 | 0.47 |
| Accession-93 | 876.67 | 310.00 | 9.50 | 2.17 | 216.67 | 24.00 | 0.58 |
| Accession-101 | 776.67 | 333.33 | 9.21 | 1.93 | 150.00 | 27.53 | 0.42 |
| Accession-103 | 816.67 | 236.67 | 9.90 | 1.00 | 156.67 | 23.33 | 0.39 |
| $LSD_{0.01}$ | 80.19 | 64.57 | 1.77 | 0.48 | 63.96 | 1.81 | 0.11 |
| Level of significance | ** | ** | ** | ** | ** | ** | ** |

** = Significant at 1% level of probability.

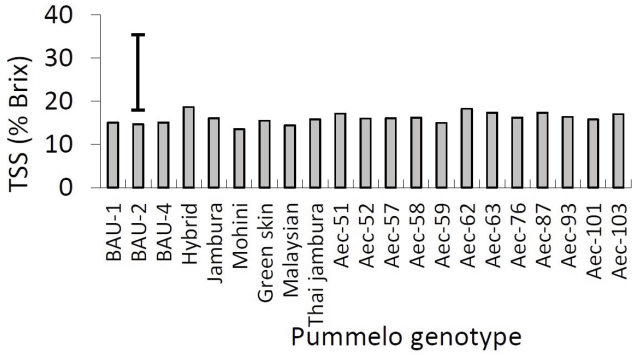



Fig. 3 - Total soluble solids (TSS) of pummelo genotype. Vertical bar indicates LSD value at 1% level of probability.

The number of fruits/plant varied significantly and ranged from 9.20 to 47.16 with the mean value of 20.88. Among the genotype Jambura (seeded) bears the maximum number of fruits (47.16) followed by Mohini (35.00) and BAU-4 (33.33) whereas Accession-62 (9.20) bears the minimum number of fruits (Table 3). In respect of number of seeds/fruit it was observed that different genotype differed significantly. The maximum number of seeds per fruit was recorded in the genotype Hybrid (114) followed by Seeded Jambura (110), BAU-4 (108.67) whereas the minimum number of seeds was found in Accession-58 (10) (Fig. 4).

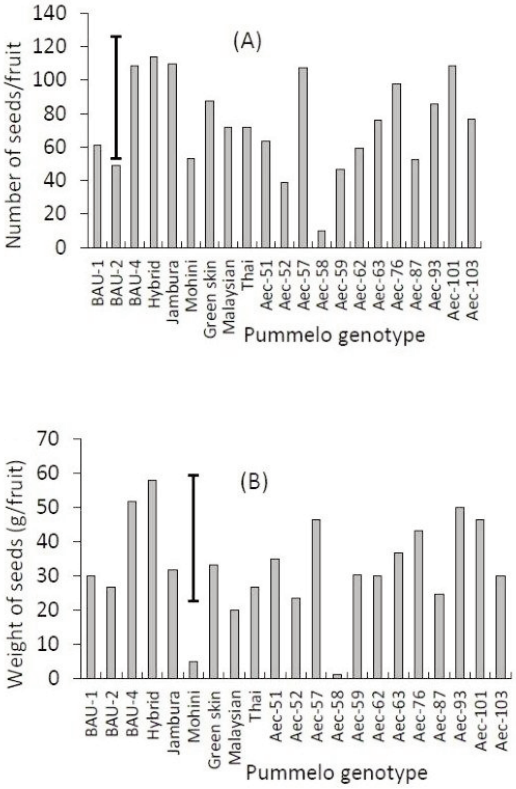



Fig. 4 - Number of seeds per fruit (A) and weight of seeds per fruit (B) of pummelo genotype. Vertical bars indicates LSD at 1% level of probability.

Like number of seeds, seed weight/fruit was also differed significantly from genotype to other genotype. The maximum weight of seeds/fruit was found in the genotype Hybrid (58 g) followed by BAU-4 (51.67 g), Accession-93 (50.00 g) whereas the minimum weight of seeds was found in the Accession-58 (1.00 g) (Fig. 5). Average weight of seed differed significantly ranging from 0.06 to 0.65 g with the mean value of 0.42 g. The heaviest average weight of seed (0.65 g) was found from the Accession-59 whereas Accession-58 gave the lowest average seed weight (0.06 g) (Table 3).

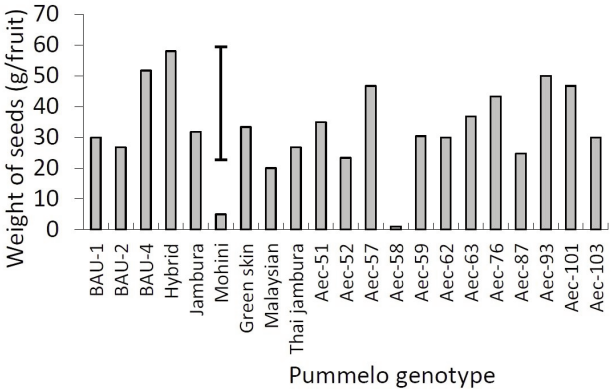



Fig. 5 - Weight of seeds per fruit of pummelo genotype. Vertical bar indicates LSD at 1% level of probability.

*Correlation coefficient*

Estimation of simple correlation coefficient was made among some important fruit producing characters of 21 pummelo genotype. The values of 'r' and the characters correlated are presented in Table 4. It was observed that leaf length had highly significant positive correlation with leaf breadth (r= 0.853**) and significant positive association with petiole wing length (0.519*) and petiole wing breadth (0.544*). It had also significant negative correlation with number of fruits/plant (-0.464*) (Table 4). Leaf breadth had highly significant positive correlation with wing breadth (0.584**) and significant positive correlation with wing length (0.450*). On the other hand, this character had negative correlation with %TSS, number of seeds, weight of seed and average weight of seed (Table 4). Correlation coefficient revealed that petiole wing length had highly positive significant correlation with petiole wing breadth (0.846**) and number of fruits/plant (0.773**). On the other hand, this character had negative correlation with weight of non-edible portion, pulp to peel ratio, average weight of seed and %TSS (Table 4). It was observed that wing breadth had positive correlation with num-

ber of anthers, thickness of pulp, total soluble solids, number of seeds/fruit and number of fruits/plant. On the other hand, this character had negative correlation with weight of fruit, weight of non-edible portion, pulp to peel ratio, average weight of seed and weight of seeds/fruit (Table 4). It was observed that number of anther had simply positive correlation with weight of fruit and weight of non-edible portion. This character had highly negative significant correlation with number of fruits/plant (-0.686**) and had negative significant correlation with number of seeds/fruit (-0.455*) (Table 4).

Correlation coefficient revealed that weight of fruit had highly positive significant correlation with weight of non-edible portion (0.818**) and thickness of pulp (0.930**). On the other hand, this character had negative significant correlation with %TSS (-0.487*) (Table 4). It was observed that weight of non-edible portion had highly positive significant correlation with thickness of pulp (0.671**) and number of fruits/plant (0.655**). On the other hand, this character had only negative correlation with %TSS (Table 4). Correlation coefficient revealed that pulp to peel ratio had simply positive correlation with thickness of pulp, %TSS, number of seeds and weight of seed/fruit. This character had negative correlation with average weight of seed and number of fruits/plant (Table 4). It was observed that thickness of pulp had significant positive correlation with number of seeds/fruit (0.440*) and had significant negative correlation with %TSS (-0.452*) (Table 4). Correlation coefficient revealed that average weight of seed had highly positive significant correlation with weight of seeds/fruit (0.588**) and number of fruits/plant (0.605**). This character had simply positive association with number of seeds/fruit (Table 4). It was observed that TSS had highly positive significant correlation with number of fruits/plant (0.823**) and had simply negative association with number of seeds/fruit (Table 4). Correlation coefficient revealed that number of seeds/fruit had highly positive significant with total weight of seed (0.822**) and had highly negative significant with number of fruits/plant (Table 4).

*Path coefficient analysis*

A path coefficient is simply a standardized partial regression coefficient and as such measures of the influence of one variable upon another permits separation of correlation coefficient into components of

Table 4 - Correlation coefficient between fruits per plant and fruit producing characters in pummelo genotype

| Traits | Leaf breadth (cm) | Petiole wing length (cm) | Petiole wing breadth (cm) | Number of anthers/ flower | Weight of fruit (g) | Weight of non-edible portion (g/fruit) | Pulp to peel ratio | Thickness of pulp (cm) | Average weight of seed (g/fruit) | TSS (% Brix) | Number of seeds/ fruit | Weight of seeds (g/fruit) | No. of fruits/ plant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf length (cm) | 0.853** | 0.519* | 0.544* | 0.176 | 0.011 | -0.043 | 0.236 | 0.071 | -0.393 | -0.093 | 0.18 | -0.052 | -0.464* |
| Leaf breadth (cm) | | 0.450* | 0.584** | 0.292 | 0.121 | 0.169 | 0.301 | 0.132 | -0.39 | -0.151 | -0.097 | -0.18 | 0.192 |
| Petiole wing length (cm) | | | 0.846** | 0.259 | 0.045 | -0.15 | -0.102 | 0.151 | -0.095 | -0.161 | 0.231 | 0.069 | 0.773** |
| Petiole wing breadth (cm) | | | | 0.227 | -0.002 | -0.208 | -0.128 | 0.082 | -0.099 | 0.014 | 0.051 | -0.018 | 0.121 |
| Number of anthers/flower | | | | | 0.021 | 0.024 | -0.334 | -0.035 | -0.01 | -0.242 | -0.455* | -0.414 | -0.686** |
| Weight of fruit (g) | | | | | | 0.818** | 0.195 | 0.930** | 0.143 | -0.487* | 0.413 | 0.387 | 0.208 |
| Weight of non-edible portion (g/fruit) | | | | | | | 0.195 | 0.671** | 0.1 | -0.298 | 0.26 | 0.308 | 0.655** |
| Pulp to peel ratio | | | | | | | | 0.235 | -0.059 | 0.079 | 0.162 | 0.234 | -0.106 |
| Thickness of pulp (cm) | | | | | | | | | 0.094 | -0.452* | 0.440* | 0.351 | 0.056 |
| Average wt. of seed (g/fruit) | | | | | | | | | | 0.306 | 0.152 | 0.588** | 0.605** |
| TSS (% Brix) | | | | | | | | | | | -0.114 | 0.139 | 0.823** |
| Number of seeds/fruit | | | | | | | | | | | | 0.822** | -0.754** |
| Weight of seeds (g/fruit) | | | | | | | | | | | | | -0.822** |

* = indicates 5% level of significance (using mean values).
** = indicate 1% level of significance (using mean values).

direct and indirect effects. The simple correlation values were used to compute the path coefficient analysis. Direct and indirect effects of different characters on fruits per plant have been presented in Table 5.

## 4. Discussion and Conclusions

The physio-morphological features of 21 pummelo genotype were found significantly different among each other. The variations were observed in leaves, flowers, fruits and seeds of all genotype. A wide variation of morphological characters of trees, leaves, fruits and seeds were identified among the pummelo clones (Paudyal and Haq, 2008). Hossain (1983) noticed varied leaf length, breadth, wing length and breadth of citrus. However, minor differences were observed between genotypic (4.043 cm) and phenotypic (4.542 cm) variance as well as genotypic (20.10%) and phenotypic (21.30%) coefficient of variation indicating minimum environmental effect upon the expression of this character. Heritability in broad sense ($H^2b$) was also calculated and it was moderately high as 89.01% (Table 6). In case of leaf breadth,

Table 5 - Path coefficient between fruits per plant and fruit producing characters in pummelo genotype

| Traits | Leaf length (cm) | Leaf breadth (cm) | Petiole wing length (cm) | Petiole wing breadth (cm) | Number of anthers/ flower | Weight of fruit (g) | Weight of non-edible portion (g/fruit) | Pulp to peel ratio | Thickness of pulp (cm) | Average weight of seeds (g/fruit) | TSS (% Brix) | Number of seeds/ fruit | Weight of seeds (g/fruit) | Number of fruits/ plant |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Leaf length (cm) | **-0.660** | 0.312 | 0.613 | -0.940 | -0.044 | 0.032 | 0.069 | -0.091 | -0.135 | -0.366 | -0.062 | 0.647 | 0.161 | -0.464* |
| Leaf breadth (cm) | -0.270 | **0.650** | 0.531 | -0.101 | -0.073 | 0.350 | -0.272 | -0.116 | -0.253 | -0.363 | -0.100 | -0.349 | 0.558 | 0.192 |
| Petiole wing length (cm) | -0.380 | 0.640 | **0.180** | -0.146 | -0.065 | 0.130 | 0.242 | 0.039 | -0.289 | -0.088 | -0.107 | 0.831 | -0.214 | 0.773** |
| Petiole wing breadth (cm) | -0.145 | 0.213 | 0.999 | **-0.173** | -0.057 | -0.006 | 0.335 | 0.049 | -0.157 | -0.092 | 0.009 | 0.184 | 0.056 | 0.121 |
| Number of anthers/flower | -0.468 | 0.107 | 0.306 | -0.392 | **-0.252** | 0.061 | -0.038 | 0.128 | 0.066 | -0.009 | -0.160 | -0.163 | 0.128 | -0.686** |
| Weight of fruit (g) | -0.029 | 0.441 | 0.053 | 0.004 | -0.005 | **0.289** | -0.132 | -0.075 | -0.177 | 0.133 | -0.322 | 0.149 | -0.120 | 0.208 |
| Weight of non-edible portion (g/fruit) | 0.114 | 0.616 | -0.177 | 0.359 | -0.006 | 0.237 | **-0.161** | -0.075 | -0.128 | 0.093 | -0.197 | 0.935 | -0.955 | 0.655** |
| Pulp to peel ratio | -0.628 | 0.109 | -0.121 | 0.229 | 0.084 | 0.564 | -0.314 | **-0.385** | -0.449 | -0.055 | 0.052 | 0.583 | -0.726 | -0.106 |
| Thickness of pulp (cm) | -0.188 | 0.481 | 0.178 | -0.141 | 0.009 | 0.269 | -0.108 | -0.090 | **-0.191** | 0.087 | -0.299 | 0.157 | -0.108 | 0.056 |
| Average weight of seeds (g/fruit) | 0.105 | -0.142 | -0.112 | 0.171 | 0.003 | 0.414 | -0.161 | 0.023 | -0.179 | **0.930** | 0.193 | -0.457 | -0.182 | 0.605** |
| TSS (% Brix) | 0.247 | -0.551 | -0.190 | -0.024 | 0.061 | -0.141 | 0.480 | -0.030 | 0.864 | 0.285 | **0.663** | -0.410 | -0.431 | 0.823** |
| Number of seeds/fruit | -0.479 | -0.354 | 0.473 | -0.088 | 0.114 | 0.119 | -0.418 | -0.062 | -0.842 | 0.752 | -0.075 | **0.361** | -0.255 | -0.754** |
| Weight of seeds (g/fruit) | 0.138 | -0.656 | 0.082 | 0.031 | 0.104 | 0.112 | -0.496 | -0.090 | -0.671 | 0.546 | 0.092 | 0.296 | **-0.310** | -0.822** |

Residual effetct: 0.1579, Bold and underlined direct effect.

Table 6 - Genotypic variance, phenotypic variance, genotypic coefficient of variation, phenotypic coefficient of variation and per cent heritability of fruit producing characters of 21 pummelo genotype

| Traits | Genotypic variance | Phenotypic variance | Genotypic CV | Phenotypic CV | Heritability (%) |
|---|---|---|---|---|---|
| Leaf length (cm) | 4.043 | 4.542 | 20.10 | 21.30 | 89.01 |
| Leaf breadth (cm) | 2.004 | 2.103 | 22.61 | 23.16 | 95.29 |
| Petiole wing length (cm) | 0.362 | 0.441 | 27.46 | 30.30 | 82.09 |
| Petiole wing breadth (cm) | 0.651 | 0.771 | 44.25 | 48.16 | 84.44 |
| Number of anthers/flower | 30.870 | 38.432 | 15.06 | 16.80 | 80.32 |
| Weight of fruit (g) | 66033.637 | 67396.887 | 31.73 | 32.06 | 97.98 |
| Weight of non-edible portion (g/fruit) | 8092.547 | 8976.507 | 30.80 | 32.44 | 90.15 |
| Pulp to peel ratio | 0.350 | 0.406 | 23.28 | 25.08 | 86.20 |
| Thickness of pulp (cm) | 2.218 | 2.884 | 15.97 | 18.21 | 76.91 |
| Average weight of seed (g/fruit) | 0.021 | 0.024 | 35.01 | 37.40 | 87.67 |
| TSS (% Brix) | 1.436 | 1.890 | 7.47 | 8.56 | 75.98 |
| Number of seeds/fruit | 754.160 | 804.840 | 37.21 | 38.44 | 93.70 |
| Weight of seeds (g/fruit) | 195.793 | 209.793 | 43.17 | 44.69 | 93.33 |
| Number of fruits/plant | 103.963 | 104.635 | 48.84 | 49.00 | 99.36 |

petiole wing length and petiole wing breadth little differences were observed between genotypic (2.004 cm, 0.362 cm, 0.651 cm) and phenotypic variances (2.103 cm, 0.441 cm, 0.771 cm) as well as genotypic (22.61%, 27.46%, 44.25%) and phenotypic coefficient of variation (23.16%, 30.30%, 48.16%) indicating minimum environmental effect upon the expression of these characters. Heritability in broad sense ($H^2b$) was also calculated and it was high as 95.29%, 82.09%, 84.44% (Table 6). Flower characters of different pummelo genotype were also varied significantly in this experiment. Hoque (2015) reported that petals number of pummelo genotype ranged from 4 to 4.5. Considerable differences were observed between genotypic (30.870) and phenotypic (38.432) variance as well as genotypic (15.06%) and phenotypic (16.80%) coefficient of variation indicating considerable environmental effect upon the expression of this character. $H^2b$ was also calculated and it was very high as 80.32% (Table 6).

Regarding fruit weight, it was found that fruit weight varied significantly among the pummelo genotype. Rahman *et al*. (2003) reported that fruit weight of local pummelo accessions varied from 718.33 g to 2160 g. While Hays (1966) noticed that the pummelo fruit is larger than the other important commercial citrus species, somewhat weighing more than 1 kg. Purseglove (1968) also observed that the fruit of pummelo is large to very large. The weight of pummelo fruit were ranged from 250 to 1218.75 g (Hossain, 1983). In Bangladesh, weight of fruit varied from 396 to 1418 g. This variation might be due to genetical, physiological, nutritional or environmental influences. The genotypic and phenotypic variances of weight of fruits were 66033.637 g and 67396.887 g, respectively. The genotypic coefficient of variation (31.73%) was lower than phenotypic coefficient of variation (32.06%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was also calculated and it was 97.98% which was high.

The genotypic and phenotypic variances of weight of non-edible portion were 8092.547 g and 8976.507 g, respectively. The genotypic coefficient of variation (30.80%) was lower than phenotypic coefficient of variation (32.44%), which indicated more influence of environment on the performance of particular trait. Heritability in broad sense ($H^2b$) was also calculated and it was 90.15%, which was moderately high. The genotypic and phenotypic variances of ratio of pulp and peel were 0.350 and 0.406, respectively. The genotypic coefficient of variation (23.28%) was lower than the phenotypic coefficient of variation (25.08%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was also calculated and it was 86.20%, which was very high.

The genotypic and phenotypic variances of thickness of pulp were 2.218 cm and 2.884 cm, respectively. The genotypic coefficient of variation (15.97%) was lower than the phenotypic coefficient of variation (18.21%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was calculated and it was 76.91%, which was very high. TSS content significantly varied among the genotype. But little differences were observed between genotypic (1.436%) and phenotypic (1.890%) variances as well as genotypic (7.47%) and phenotypic (8.56%) coefficient of variation indicating low environmental influence on this trait. $H^2b$ was calculated and it was 75.98% which was high. The number of fruits/plant varied significantly among the genotype. Verheij and Coronel (1992) reported that a single tree can yield 70-100 fruits/year. The genotypic and phenotypic variances of thickness of number of fruits/plant were 103.963 and 104.635, respectively. The genotypic coefficient of variation (48.84%) was lower than the phenotypic coefficient of variation (49.00%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was also calculated and it was 99.36% which was very high.

Maximum number of seeds/fruit was observed in Hybrid (114) among the studied genotype and minimum seed number was found in Accession-58. Hossain (1983) reported that the number of seeds in pummelo fruits varied from 8 to 94/fruit and also he found both seedless and seeded genotype. The genotypic and phenotypic variances of number of seeds/fruit were 754.160 and 804.840, respectively. The genotypic coefficient of variation (37.21%) was lower than the phenotypic coefficient of variation (38.44%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was also calculated and it was 93.70% which was very high. The genotypic and phenotypic variances of total weight of seeds were 195.793 g and 209.793 g, respectively. The genotypic coefficient of variation (43.17%) was lower than the phenotypic coefficient of variation (44.69%), which indicated more influence of environment on the performance of particular trait. $H^2b$ was also calculated and it was 93.33%, which was very high.

Correlation coefficient revealed that leaf breadth, petiole wing length and breadth will be increased with the increase of leaf length. On the other hand, this trait had negatively associated with weight of non-edi-

ble portion, average weight of seeds/fruit, %TSS and weight of seeds/fruit. This indicate that petiole wing length and breadth will be increased with the increase of leaf breadth. On the other hand, this trait had negatively associated with TSS, number of seeds/fruit and total weight of seeds/fruit. Correlation coefficient revealed that petiole wing breadth and number of fruits/plant will be increased with the increase of petiole wing length. Correlation coefficient revealed that weight of non-edible portion and thickness of pulp will be increased with the increase of fruit weight. This indicate that thickness of pulp and number of fruits/plant will be increased with the increase of weight of non-edible portion. On the other hand, this trait had negatively associated with %TSS. It was observed that Correlation coefficient revealed that thickness of pulp indicates that number of seeds/fruit will be increased with the increase of thickness of pulp. Correlation coefficient revealed that average weight of seed/fruit indicates total weight of seeds and number of fruits/plant will be increased with the increase of average weight of seed.

Correlation coefficient revealed that %TSS indicates number of fruits/plant will be increased with the increase of %TSS. Correlation coefficient revealed that number of seeds/fruit indicates weight of seeds/fruit will be increased with the increase of number of seeds/fruit. Leaf length showed low direct negative effect (-0.66) on fruits/plant whereas it also contributed indirect positive effect via leaf breadth, petiole wing length, fruit weight, weight of non-edible portion, number of seeds/fruit and weight of seeds/fruit. Leaf breadth contributed low direct positive effect (0.65) on fruits/plant. This trait had also indirect positive effect on wing length, fruit weight, weight of seeds/fruit and number of fruits/plant. Petiole wing length showed low direct positive effect (0.18) on fruits/plant whereas it also contributed indirect positive effect on fruits/plant via leaf breadth, fruit weight, weight of non-edible portion, pulp to peel ratio and number of seeds/fruit.

Petiole wing breadth contributed low direct negative effect (-0.173) on fruits/plant. This trait had also indirect positive effect on number of fruits/plant via leaf breadth, petiole wing length, weight of non-edible portion, pulp to peel ratio, %TSS, number of seeds/fruit and weight of seeds per fruit. Number of anthers/flower showed low direct negative effect (-0.252) on fruits/plant. This trait had also indirect positive effect on leaf length, wing length, weight of fruit, pulp to peel ratio, thickness of pulp and weight of seeds/fruit. Fruit weight showed low positive effect (0.289) on fruits/plant whereas it also contributed indirect positive effect on number of fruits/plant via leaf length, leaf breadth, wing length, average weight of seed and number of seeds/fruit. Weight of non-edible portion contributed direct negative effect (-0.161) on fruits/plant. This trait had also indirect positive effect on number of fruits/plant via leaf length, leaf breadth, petiole wing breadth, weight of fruit, average weight of seed and number of seeds/fruit.

Pulp to peel ratio showed direct negative effect (-0.385) on fruits/plant whereas it also contributed indirect positive effect via leaf breadth, petiole wing breadth, number of anthers/flower, weight of fruit, %TSS and number of seeds/fruit. Thickness of pulp contributed direct negative effect (-0.191) on fruits/plant. This trait had also indirect positive effect on number of fruits/plant via leaf breadth, petiole wing length, weight of fruit, number of anthers, average weight of seeds and number of seeds/fruit. Average weight of seeds/fruit showed direct positive effect (0.93) on fruits/plant whereas it also contributed indirect positive effect on fruits/plant via leaf length, petiole wing breadth, number of anthers/flower, fruit weight, pulp to peel ratio and TSS. TSS showed direct positive effect (0.663) on fruits/plant. This trait had also indirect positive effect on number of fruits/plant via leaf length, number of anthers/flower, weight of non-edible portion, thickness of pulp and mean weight of single seed. Number of seeds/fruit contributed direct positive effect (0.361) on fruits/plant whereas it also contributed indirect effect via petiole wing length, number of anthers/flower, weight of fruit and average weight of seeds/fruit. Weight of seeds/fruit showed direct negative effect (-0.31) on fruits/plant. This trait had also indirect positive effect via leaf length, petiole wing length, petiole wing breadth, number of anthers/flower, weight of fruit, average weight of seeds/fruit, TSS (%) and number of seeds/fruit.

It was noticed that a wide variations existed among the pummelo genotype and these variability could be useful for varietal improvement of pummelo in Bangladesh. It appeared from the study that grouping of the genotype differed based on morphological traits. In respect on different traits genotype hybrid was found potential followed by BAU-1.

**References**

BBS, 2015 - *Monthly statistical bulletin*. - Bangladesh

Bureau of Statistics, Statistics Division, Ministry of planning, Government of People's Republic of Bangladesh, Dhaka, Bangladesh, pp. 144.

BHATT G.M., 1973 - *Significance of path coefficient analysis in determining the nature of character association*. - Euphytica, 22(2): 338-343.

BURTON G.W., 1952 - *Quantitative inheritance in grasses*. - Proceedings of 6th International Grassland Congress, 1: 277-283.

DEWEY D.R., LU K.H., 1959 - *A correlation and path-coefficient analysis of component of crested wheatgrass seed production.* - Agron. J., 51: 515-518.

GOMEZ K.A., GOMEZ A.A., 1984 - *Statistical procedure for agricultural research*. - 2nded., John Willey and Sons, New York, USA, pp. 28-192.

HAYES W.B., 1966 - *Fruit growing in India.* - 3rd ed., Allahabad Agricultural Institution, Allahabad, India, pp. 209.

HOQUE M.A., 2015 - *Floral biology of indigenous pummelo genotypes*. - Bangladesh J. Agril. Res., 40(2): 177-188.

HOSSAIN M.M., 1983 - *Morphological studies of different citrus plants*. - M. SC. (Ag.) thesis, Department of Horticulture, BAU, Mymensingh, Bangladesh, pp. 112.

IPGRI, 1999 - *Descriptors for citrus*. - International Plant Genetic Resources Institute, Rome, Italy, ISBN 92-9043-425-2.

JOHNSON H.W., ROBINSON H.F., COMSTOCK R.E., 1955 - *Estimates of genetic and environmental variability in Soybeans*. - Agron. J., 47: 314-418.

MORTON J.F., 1987 - *Pummelo*, pp. 147-151. - In: DOWLING C.F. (ed.) *Fruits of warm climates*. Florida Flair Books, Miami, FL, USA, pp. 505.

PAUDYAL K.P., HAQ N., 2008 - *Variation of pomelo (*Citrus grandis *L. Osbeck) in Nepal and participatory selection of strains for further improvement.* - Agroforst. Syst., 72(3): 195-204.

PURSEGLOVE J.W., 1968 - *Tropical crops dicotyledon*. - Lonmans Green and Company Limited, London, UK, pp. 502.

RAHMAN M.M., RABBANI M.G., KHAN A.S.M.M.R., ARA N., RAHMAN M.O., 2003 - *Study on physio-morphological characteristics of different local pummelo accessions*. - Pakistan J. Biol. Sci., 6: 1430-1434.

RANGANNA S., 1978 - *Manual of analysis of fruit and vegetable products*. - Tata McGraw-Hill Publishing Company Limited, New Delhi, India, pp. 634.

SINGH R.K., CHAUDHARY B.D., 1985 - *Biometrical methods of quantitative genetic analysis*. - Haryana J. Hort. Sci., 12(2): 151-156.

VERDI A., 1988 - *Application of recent taxonomical approaches and new techniques to citrus breeding*. - Proceeding of the 6th International Citrus Congress, Tel Aviv, Israel, March 6-11, Vol. 2, pp. 303-315.

VERHEIJ E.W.M., CORONEL R.E., 1992 - *Plant resources of South-East Asia No. 2.* - Edible Fruits and Nuts. Prosea Foundation, Bogor, Indonesia.

Adv. Hort. Sci., 2018 32(1): 105-111 DOI: 10.13128/ahs-22807

AHS
Advances in Horticultural Science

# Volatile compounds from different fruit parts of two cultivars of *Cydonia oblonga*

**C. Taiti [1], E. Giordani [1], E. Palm [1], W.A. Petrucci [1], G. Bennati [2], G. Gestri [2], E. Marone [3 (*)], E. Azzarello [1], S. Mancuso [1]**

[1] *Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università degli Studi di Firenze, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy.*

[2] *Associazione oasi apistica le buche, Via Regina Margherita, 26, 59016 Poggio a Caiano (PO), Italy.*

[3] *Facoltà di Bioscienze e Tecnologie Agro-Alimentari e Ambientali, Università degli Studi di Teramo, Via R. Bazarini, 1, 64100 Teramo, Italy.*

*Key words:* fruit tissues, Proton Transfer Reaction Time-of-Flight Mass Spectrometer, physicochemical fruit parameters, quince, VOCs.

**Abstract: Quince is characterized as a fragrant fruit which, unlike other pomes (apple, pear), is not used for fresh consumption due to its astringency and compactness, but only in its processed form (jams, jelly, distillery products, and nutraceutical compounds). As a consequence, there is little knowledge currently available concerning the characteristics of the fruit, and in particular its aromatic and chemotaxonomic patterns. In this work, carpometric, chemometric and spectrophotometric measurements were performed on quince fruits. VOCs emitted by different tissues or parts of the fruit were studied to describe its aromatic profile. The study was carried out on the fruits of an old, well-known cultivar ('Gigante di Wranja', commonly called 'Wranja') and a new Tuscan accession. Intact, halved and solely pulp (cubed) samples were evaluated for each individual fruit. Data obtained from VOC analysis through Proton Transfer Reaction Time-of-Flight Mass Spectrometer (PTR-ToF-MS) were evaluated by multivariate statistical analysis. The spectra obtained from the intact fruit samples showed a higher amount of masses corresponding to terpenes or terpenoid compounds, which fundamentally characterize the aroma of this type of fruit; these substances were found to be much less present in the VOCs emitted by the pulp, where high values of masses linked to the maturation processes were instead found.**



(*) **Corresponding author:**
emarone@unite.it

**Citation:**
TAITI C., GIORDANI E., PALM E., PETRUCCI W.A., BENNATI G., GESTRI G., MARONE E., AZZARELLO E., MANCUSO S., 2018 - *Volatile compounds from different fruit parts of two cultivars of Cydonia oblonga.* - Adv. Hort. Sci., 32(1): 105-111



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 2 December 2017
Accepted for publication 6 March 2018

## 1. Introduction

Plants are not only a dietary source for both human beings and animals but also serve as medicinal, nutritional, and ornamental purposes

(Ashraf *et al.*, 2016). The potential of plants as medicine is supported by abundant scientific evidence (Lattanzio *et al.*, 2009) and researchers are currently focused on isolating new aromas and active phytochemicals produced by different plant organs. Quince is the fruit of an ancient tree, *Cydonia oblonga* Miller of the Rosaceae family, and has a very wide origin area that ranges from Anatolia to the Caucasus. It was known to the Greeks and Romans, and it seems that the name of the genus *Cydonia* comes from the ancient name of the town of Chania on the island of Crete (Roversi, 1991). It is a climacteric, large-sized (up to 500 g) fruit, namely pome (false fruit), and generally oblong (pyriform types) or rounded (apple-shared types) (Bellini *et al.*, 2007). The harvest is still carried out by hand, although the desire to mechanize this operation with systems similar to those used for the mechanical harvesting of the apple tree is increasing (Fiorino *et al.*, 2010). When ripening, the color of the fruit epidermis turns from greenish-yellow to bright yellow when fully ripe. The pulp is very firm, and not directly edible due to the presence of tannins and pectic substances (Bellini *et al.*, 2007). Ripe fruits are commonly used as a source of pectin, and to produce jam or jelly, liquors, and distilled products with aromatic notes typical of quince (Silva *et al.*, 2004). The fruits can also be beneficial to human health thanks to their high phytochemical content (Ashraf *et al.*, 2016). Many studies have reported *Cydonia oblonga* as an excellent natural source of phenolic and flavonoid compounds which are considered potent antioxidants (Silva *et al.*, 2004; Oliveira *et al.*, 2007). Quince fruit is considered an important dietary source of health-promoting compounds due to its antioxidant, antimicrobial and antiulcerative properties (Magalhães *et al.*, 2009).

Although the aroma is one its most important fruit-derived parameters, only a few studies have focused on the volatile emission by *Cydonia* (Tateo and Bononi, 2010), and in particular, by different tissues of the fruit. Indeed, the intense and pleasant aroma released by *Cydonia* may represent an important starting point in the genetic improvement of this species.

Recently, Proton Transfer Reaction-Time of Flight-Mass Spectrometry (PTR-ToF-MS) was proposed as an innovative analytical technology for volatile organic compound (VOC) detection and quantification on fruit matrices due to its capacity to rapidly provide a comprehensive mass spectrum with high-time resolution and without sample treatment (Mayr *et al.*, 2003; Taiti *et al.*, 2017 a). The aim of the current study was to analyze the aroma profile of different fruit parts of two *Cydonia oblonga* genotypes, both grown in Italy, to increase the chemotaxonomic knowledge, fruit management and quince product trade.

## 2. Materials and Methods

*Plant materials*

Quince fruits belonging to an old, well-known cultivar ('Gigante di Wranja', commonly called 'Wranja') and a new Tuscan accession were harvested in Tuscany (Italy) in the last week of September 2017. For each cultivar, 12 homogeneous and healthy fruits were selected, gently brushed to eliminate the hair cover and washed in deionized water. Fruits were stored in a climatic chamber (14±1°C, 85-90% relative humidity) for three days prior to laboratory analysis. For all the fruits, color, weight, maximum diameter and height were determined. Six fruits were used for the spectrophotometric tests by PTR-ToF-MS and the remaining six for the physicochemical measurements. For chemical analyses (Lugol starch test and refractometric grade), fruits were divided in half so that one half of each fruit was used for the starch test and the other to evaluate refractometric grade.

*Color and physicochemical fruit parameters*

*Peel color.* The identification of the different peel color of quince fruits was assessed using a Minolta CR-200 Chroma-Meter (Minolta, Ramsey, NJ, USA) according to the Hunter scale, as previously described by Taiti *et al.* (2017 b).

*Fruits dimensions and firmness.* For each cultivar, the weight (g), maximum diameter (cm), and height (cm) of twelve samples were measured. The firmness of each fruit (expressed as kgf) was measured as the force needed to reduce fruit diameter by 2 mm using a penetrometer (Model 53205D, Turoni, Italy).

*Total soluble solids (TSS).* To evaluate TSS concentration, six quince halved fruits for each genotype were squeezed to collect a few drops of juice; then TSS levels were measured with an N1 Atago refractometer (Atago Co., Japan) and expressed as °Brix.

*Starch Iodine Test.* Starch-iodine test (Lugol solution) was performed by visual evaluation on halved fruits and by scoring samples on a 'Golden Delicious' standardized 1-9 scale (Smith *et al.*, 1979).

*Volatile compounds detection*

VOCs analyses were carried out with a high-reso-

lution PTR-ToF-MS (IONICON Analytik GmbH, Austria) using $H_3O^+$ as ion donor. For each genotype, six fruits were used; the same fruit was first analyzed as intact whole, then divided into two parts, analyzing the halves together; finally, two cubes were got from each one of the halved fruit respectively, without peel (5 × 5 × 5 cm, about 10 g each one); all the measurements were performed in triplicates on the same sample. All whole and halved samples were weighed, and the VOCs data were based on 100 g samples for whole and halved samples to allow for comparisons between the obtained results.

The drift tube of PTR-ToF-MS instrument was set to 80°C and operated with a drift pressure of 2.30 mbar and a voltage of 550 V. These settings lead to an E/N ratio (E, electric field strength in the drift tube; N, buffer gas number density in the drift tube) equal to 135 Townsends (1 Td = 1017 $Vcm^2$). Mass/charge ratio of peaks detected at *m/z* 21 (signal for $H_3O^+$) and *m/z* 37 [signal for water clusters $(H_2O)H_3O^+$] were monitored during all measurements to check the instrument's stability and cluster ion formation. To reach a good mass accuracy (up to 0.001 Th), internal calibration was based on three points and was performed off-line. Acquisition and post-processing data were performed as reported by Marone *et al.* (2017).

*Statistical analyses*

One-way analyses of variance (ANOVA) were performed (1) to compare the physicochemical parameters of two quince genotypes, and (2) to compare the VOC emission profiles whole, halved, and cubed fruit samples. Separation of means was performed by the Fisher's LSD test (p= 0.05). Computations were performed by Statgraphics Centurion XV v. 15.0.04. A Principal Component Analysis (PCA) was applied to the whole spectral data of 36 quince samples, previously submitted to a logarithmic transformation and mean centering as pre-processing. Computations were performed by PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB_ R2015b (Mathworks Inc., Natick, MA, USA). A Correspondent Analysis (CA) was applied to the spectral data of the 36 quince samples to build up a simultaneous ordination of quince samples and protonated *m/z*, thus facilitating the evaluation of their reciprocal relationships. The analysis preserves the $X^2$-distances between the data matrix rows and columns. Data were weighted using the symmetric option (module HIERCLUS, SYN-TAX 2000 program package).

## 3. Results and Discussion

*Physicochemical analyses*

The results of the physicochemical analyses on the different cultivars are reported in Table 1, and are largely in agreement with a previous study on different cultivars (Leonel *et al.*, 2016). Among the physicochemical parameters analyzed, some significant differences were observed between the two *Cydonia* accessions, with regard to firmness, °Brix values and starch contents. In particular, the °Brix content was lower for 'Wranja' (12.2±0.9) compared to Tuscan accession (17.2±0.9); vice versa the starch content was higher for 'Wranja'. The starch and sugar contents measured for the Tuscan accession indicate that these fruits may have been further along in the ripening process than the 'Wranja' fruits.

*VOCs headspace analysis*

Samples (whole, halved and cubed) belonging to two different *Cydonia* genotypes were analyzed by PTR-ToF-MS detecting a total of 67 peak signals (Fig. 1). These signals represent different groups of volatile compounds including hydrocarbons, esters, alcohols, terpenoids, aldehydes, ketones and lactones. These results show that, even if no different volatile compounds were identified, different emission intensities were found between the two genotypes (Fig. 1). On the other hand, among fruit tissues, both differences in number and emission intensity of each detected compound were instead observed. Figure 1 shows that for both cultivars, the intensity and number of compounds emitted were higher in

Table 1 - One-way analysis of variance (ANOVA) related to the quince fruits physicochemical parameters (average ± sd)

| Cydonia accessions | Weight (g) | Ø (mm) | High (mm) | Firmness (kgf) | °Brix | Starch | Overcolor | | | Background color | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | L* | A* | B* | L* | A* | B* |
| Wranja | 268.9 | 82.8 | 77.8 | 6.1 | 12.2 | 4.25 | 77.7 | -12.5 | 62.6 | 72.6 | -15.9 | 58.4 |
| | ±54.5 | ±7.0 | ±4.5 | ±0.9 a | ±0.9 a | ±0.5 a | ±2.4 | ±2.4 | ±3.3 | ±4.1 | ±2.3 | ±3.1 |
| Tuscan | 320 | 86.5 | 85.4 | 7.6 | 17.2 | 2.75 | 76.9 | -13.1 | 57.6 | 76.6 | -12.8 | 60 |
| | ±92.6 | ±9.2 | ±9.7 | ±0.4 b | ±0.9 b | ±0.5 b | ±4.7 | ±2.3 | ±5.6 | ±2.6 | ±2.2 | ±2.9 |

Different lowercase letters within a column indicate differences by the LSD test at the 95% confidence level (p= 0.05).

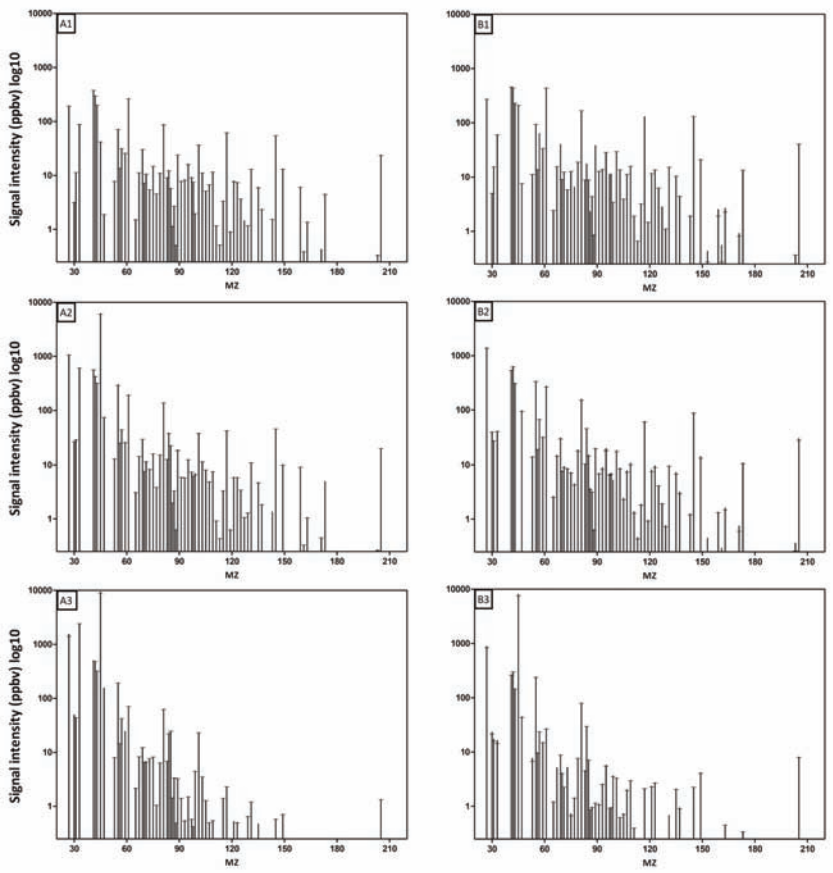



Fig. 1 - Typical PTR-MS mass spectrum (average, n=6) obtained by different fruit parts of two cultivars of *Cydonia*. (A) Tuscan accession, (B) 'Wranja'; (1) whole fruit, (2) halve fruit, (3) cube fruit.

the whole fruit followed by halved fruit, and lowest in cubed fruit. These results are in agreement with a previous study of Imayoshi *et al.* (1995) on Nashi pear, where differences in the total number of VOCs among pulp, peel, and whole fruit were found. Indeed, as reported by Paillard (1981) for many fruits species, VOC production changes among different fruit tissues, being highest in the skin and nearby tissues. It is noteworthy that fruit's aroma depends on the combination of VOCs produced, and on the concentration and odor threshold of each in the blend. A further investigation of the spectral data was performed by a multivariate ordination. The PCA (Fig. 2) approach applied to the whole dataset (ppbv) of 67 detected protonated masses (data not shown) give a general view of the quince sample ordination, based on the analysis of defined fruits parts (intact, halved, and cubed fruits) related to the two chosen accessions (Tuscan accession and 'Wranja'). The first three components accounted for 96.4% of the total variability (66.1%, 17.7%, 12.7%, respectively). A strong effect related to the type of the sample used rather than the cultivar is evident. The whole fruit samples of both cultivars, joined in the lower right quadrant of the diagram, strongly differ from those obtained from the head space of the pulp only, both correlated with the negative part of the x-axis (PC1), but clustered based on the cultivar, and respectively related to the positive part (Tuscan accession) and to the negative part (Wranja) of the y-axis (PC2). The halved fruit group is placed in the upper right quadrant, with the two cultivars partially overlapped, indi-

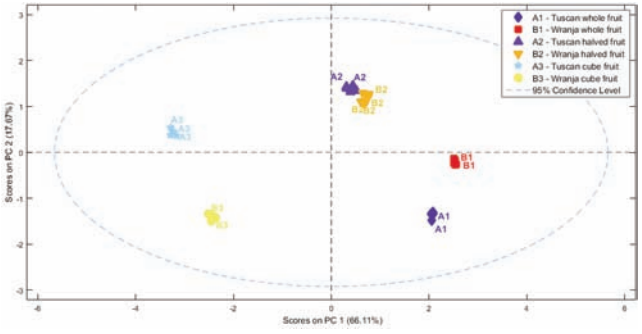



Fig. 2 - PCA scores of the quince samples based on the full spectra distribution of the 'Tuscan accession' and 'Wranja'. (A1) = 'Tuscan accession' whole fruits; (A2) = 'Tuscan accession' halved fruits; (A3) = 'Tuscan accession' cube fruits; (B1) = 'Wranja' whole fruits; (B2) = 'Wranja' halved fruits, (B3) = 'Wranja' cube fruits.

cating a greater similarity of masses with the intact whole fruit, even if positively correlated with PC2. To better evaluate the effect of specific chemical family compounds, the 20 masses with statistically significant (p= 0.05) discriminating power among the different parts of the fruit were selected by ANOVA within the whole spectrum (Table 2), upon which a Correspondence Analysis was subsequently applied.

The join plot from CA (Fig. 3) simultaneously displays quince different sample and protonated *m/z* ordinations based on the results of the variable selection. The intact fruits of both cultivars are characterized by a group of masses in which terpenes and terpenoids are well represented. In particular, the highest presence of monoterpene (*m/z* 135, *m/z* 137), terpenoid (*m/z* 153) and sesquiterpene (*m/z* 205) compounds distinguished the whole fruit from the other two types of cut samples. According to Taiti *et al.* (2017 b), the higher emission intensity and number of VOCs detected in whole fruits was associated to the presence of the peel. It is well known that a great diversity of VOCs linked to the pericarp tissue of the peel are emitted, such as esters, alcohols, aldehydes and terpenes (Chervin *et al.*, 2000; Rodriguez *et al.*, 2013).

The VOCs linked to cutting, such as the green leaf volatiles (GLVs) (i.e., hexenal, hexanal), are highest in halved and cubed fruits, compared to intact fruits where the intensities of these signals were very low (Table 2), and confirmed by the fact that these compounds were well represented in pulp tissue.

Moreover, halved fruits occupy an intermediate position, while cubed fruit samples are shifted to the right in the diagram (Fig. 3), with a differentiation along the axis of the y (axis 2) determined by the masses *m/z* = 45.033, *m/z* = 47.049 and *m/z* 33.033 (only for the Tuscan accession). As reported by Taiti *et al.* (2015) the methanol emission in fruits increase

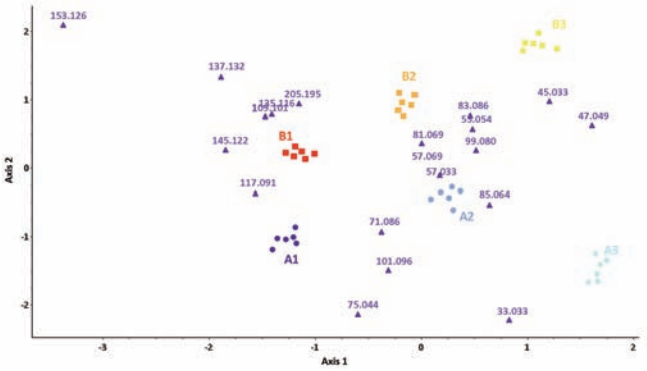



Fig. 3 - Join plot from Correspondence Analysis. (A1) = 'Tuscan accession' whole fruits; (A2) = 'Tuscan accession' halved fruits; (A3) = 'Tuscan accession' cube fruits; (B1) = 'Wranja' whole fruits; (B2) = 'Wranja' halved fruits, (B3) = 'Wranja' cube fruits. Numbers correspond to the protonated m/z.

Table 2 - Protonated selected masses discriminating whole, halved and cubed fruit samples, identified via PTR-ToF-MS: Mass/charge (m/z) ratios, chemical formula, tentative identifications, minimum and maximum values detected (ppbv) and VOC, as previously reported in the literature (Cydonia*; PTR-MS#)

| No. | Protonated *m/z* | Chemical formula | Tentative identification | Min detected value (ppbv) | Max detected value (ppbv) | References |
|---|---|---|---|---|---|---|
| 1 | 33.033 | $CH_5O^+$ | Methanol | 11.7 | 2724.1 | Khoubnasabjafari and Jouyban (2011)* |
| 2 | 45.033 | $C_2H_5O^+$ | Acetaldehyde | 35 | 16416.9 | Umano *et al.* (1986)* |
| 3 | 47.049 | $C_2H_7O^+$ | Ethanol | 1.5 | 199.8 | Umano *et al.* (1986) |
| 4 | 55.054 | $C_4H_7^+$ | C4 aldehydes fragment | 65.3 | 433.1 | Taiti *et al.* (2017 b)# |
| 5 | 57.033 | $C_3H_5O^+$ | Alkyl fragment (hexanal/hexyl acetate) | 8.5 | 25.4 | Taiti *et al.* (2017 b)# |
| 6 | 57.069 | $C_4H_9^+$ | Alkyl fragment (Hexanol/valeric acid) | 18.1 | 75.2 | Taiti *et al.* (2017 b)# |
| 8 | 75.044 | $C_4H_{11}O^+$ | 2-butanol/2-methyl-1-propanol | 0.5 | 19.7 | Umano *et al.* (1986)* |
| 9 | 81.069 | $C_6H_9^+$ | Terpenes and C6 fragments | 49.8 | 203.5 | Maleknia *et al.* (2007) # |
| 10 | 83.085 | $C_6H_{11}^+$ | C6 fragments /hexenol fragment | 8.8 | 50.7 | Maleknia *et al.* (2007)# |
| 11 | 85.064 | $C_5H_9O^+$ | 2-Methyl-2-butenal/1-Penten-3-one | 4.6 | 26.2 | Khoubnasabjafari and Jouyban (2011)* |
| 12 | 99.080 | $C_6H_{11}O^+$ | (E)-2-hexenal | 1.6 | 8.9 | Umano *et al.* (1986)* |
| 13 | 101.096 | $C_6H_{13}O^+$ | Hexanal/(Z)-3-hexen-1-ol | 1.7 | 44.8 | Tateo and Bonomi (2010)* |
| 14 | 109.101 | $C_8H_{13}^+$ | Terpene fragments | 0.4 | 23.6 | Maleknia *et al.* (2007) # |
| 15 | 117.091 | $C_6H_{13}O_2^+$ | Isobutyl acetate/Ethyl butyrate/butyl acetate | 1.5 | 176.7 | Tateo and Bonomi (2010)* |
| 16 | 135.116 | $C_{10}H_{15}^+$ | p-Cymene | 0.4 | 11.9 | Tateo and Bonomi (2010)* |
| 17 | 137.132 | $C_{10}H_{17}^+$ | Monoterpenes (e.g. limonene, g-terpinene) | 0 | 5.1 | Tateo and Bonomi (2010)* |
| 18 | 145.122 | $C_8H_{17}O_2^+$ | Ethyl hexanoate | 0.5 | 153.9 | Tateo and Bonomi (2010)* |
| 19 | 153.127 | $C_{10}H_{17}O^+$ | Oxygenated Terpenes (e.g. geranial) | 0 | 0.4 | Tsuneya *et al.* (1983)* |
| 20 | 205.195 | $C_{15}H_{25}^+$ | Sesquiterpenes (e.g. bergamotene) | 1.1 | 49.8 | Tateo and Bonomi (2010)* |

steadily throughout the ripening process. On the other hand, ethanol is the product of anaerobic metabolism and, together with acetaldehyde, accumulates in pome fruit under imposed hypoxia and poor gas exchange in the pulp tissue (Pinto *et al.* 2001). Pome fruit contains ethanol and acetaldehyde as part of their aroma (Ritenour *et al.* 1997) and as reported by Rapparini and Predieri (2003) these compounds increase together with the ripening process and their emission increased at a faster rate with the onset of fruit senescence.

## 4. Conclusions

The typical quince fruit aroma depends on a mix of volatile compounds which originate by different parts of the pome. By the analyses of different fruit tissues, differences in VOCs number and in their emission intensity were observed, while little difference was observed between the two varieties analyzed. Moreover, it has been shown that the intensity and number of compounds emitted was highest in the whole fruit, followed by halved fruit and cubed fruit. Through multivariate analysis, some masses have been highlighted that determine the differentiation of the spectral composition between the VOCs emitted by the pulp (halved and cubed fruit samples) and those obtained from intact fruit that could be specifically responsible for the particular aroma of quince fruit. The four masses referring to terpenes and terpenoids are prevalent in the peel, and are moderately related to the cultivar. Further work should be done to increase the knowledge concerning the chemotaxonomic profiles of the different cultivars, and to understand how through this tool, innovations can be developed and moved to quince genetically improved and to the food industry.

## References


ASHRAF M.U., MUHAMMAD G., HUSSAIN M.A., BUKHARI S.N., 2016 - Cydonia oblonga *M., a medicinal plant rich in phytonutrients for* pharmaceuticals. - Frontiers in pharmacology, 7: 163.

BELLINI E., GIORDANI E., GIANNELLI G., PICARDI E., 2007 - *Quince.* Cydonia oblonga *MIll*., pp. 439-457. - In: BELLINI E. (ed). *The fruit woody species*. *Descriptor List*. Arsia Regione Toscana, Press Service, Sesto Fiorentino, Firenze, Italy, pp. 1070.

CHERVIN C., SPEIRS J., LOVEYS B., PATTERSON B.D., 2000 - *Influence of low oxygen storage on aroma compounds of whole pears and crushed pear flesh*. - Postharvest Biol. Technol., 19(3):279-285.

FIORINO P., MARONE E., OTTANELLI A., 2010 - *Mechanical harvesting, productivity and superintensive planting systems in olive groves* - Adv. Hort. Sci., 24(1): 91-94.

IMAYOSHI Y., YUKAWA C., IWABUCHI H., BASER K.H.C., 1995 - *Volatile constituents of Chinese pear 'Yali'*. - Proc. 13th Int. Congress of Flavours, Fragrances and Essential Oils, Turkey, 2: 15-19.

KHOUBNASABJAFARI M., JOUYBAN A., 2011 - *A review of phytochemistry and bioactivity of quince (*Cydonia oblonga *Mill.)*. - J. Med. Plants Res., 5(16): 3577-3594.

LATTANZIO V., KROON P.A., LINSALATA V., CARDINALI A., 2009 - *Globe artichoke: a functional food and source of nutraceutical ingredients*. - J. Func. Foods, 1: 131-144.

LEONEL M., LEONEL S., TECCHIO M.A., MISCHAN M.M., MOURA M.F., XAVIER D., 2016 - *Characteristics of quince fruits cultivars (*Cydonia oblonga *Mill.) grown in Brazil.* - Australian J. Crop Sci., 10(5): 711.

MAGALHÃES A.S., SILVA B.M., PEREIRA J.A., ANDRADE P.B., VALENTAO P., CARVALHO M., 2009 - *Protective effect of quince (*Cydonia oblonga *Miller) fruit against oxidative hemolysis of human erythrocytes*. - Food and Chemical Toxicology, 47(6): 1372-1377.

MALEKNIA S D., BELL T.L., ADAMS M.A., 2007 - *PTR-MS analysis of reference and plant-emitted volatile organic compounds*. - Inter. J. Mass Spectrometry, 262(3): 203-210.

MARONE E., MASI E., TAITI C., PANDOLFI C., BAZIHIZINA N., AZZARELLO E., FIORINO P., MANCUSO S., 2017 - *Sensory, spectrometric (PTR-ToF-MS) and chemometric analyses to distinguish extra virgin from virgin olive oils*. - J. Food Sci. Technol., 54(6): 1368-1376.

MAYR D., MARK T., LINDINGER W., BREVARD H., YERETZIAN C., 2003 - *Breath-by-breath analysis of banana aroma by proton transfer reaction mass spectrometry*. - Inter. J. Mass Spectrometry, 223: 743-756.

OLIVEIRA A.P., PEREIRA J.A., ANDDRADE P.B., VALENTAO P., SEABRA R.M., SILVA B.M., 2007 - *Phenolic profile of* Cydonia oblonga *Miller leaves.* - J. Agric. Food Chem., 55: 7926-7930.

PAILLARD N.M.M., 1981 - *Factors influencing flavour formation in fruits*, pp. 479-507. - In: SCHREIER P. (Ed.) *Flavour '81*. 3rd Weurman Symposium Proceedings of the International Conference, Munich, April 28-30, Walter de Gruyter, Berlin, New York.

PINTO K., LENTHERIC I., VENDRELL M., LARRIGAUDIERE C., 2001 - *Role of fermentative and antioxidant metabolism in the induction of core browning in controlled-atmosphere stored pears*. - J. Sci. Food Agr., 81: 364-370.

RAPPARINI F., PREDIERI S., 2003 - *Pear fruit volatiles*. - Hort. Reviews, 28: 237-324.

RITENOUR M.A., MANGRICH M.E., BEAULIEU J.C., RAB A., SALTVEIT M.E., 1997 - *Ethanol effects on the ripening of climacteric fruit*. - Postharvest Biol. Technol., 12: 35-42.

RODRIGUEZ A., ALQUEZAR B., PENA L., 2013 - *Fruit aromas in mature fleshy fruits as signals of readiness for predation and seed dispersal*. - New Phytologist, 197(1): 36-48.

ROVERSI A., 1991 - *Cotogno*, pp. 164-172. - In: AVANZATO D., G. BARBERA, G. BARGIONI, E. BELLINI, A. BERGAMINI, F. CALABRESE, A. DE MICHELE, R. DI LORENZO, E. FAEDI, C. FIDEGHELLI, G. FONTANAZZA, A. GODINI, R. GUERRIERO, O. INSERO, F. MONASTRA, G. PAESANO, R. PAGLIETTA, A. ROVERSI, G. TAMPONI, and A. TOMBESI *Frutticoltura speciale*. REDA, Roma, Italy, pp. 783.

SILVA B.M., ANDRADE P.B., VALENTAO P., FERRERES F., SEABRA R.M., FERREIRA M.A., 2004 - *Quince (*Cydonia oblonga *Miller) fruit (pulp, peel, and seed) and jam: antioxidant activity*. - J. Agric. Food Chem., 52: 4405-4712.

SMITH R.B., LOUGHEED E.C., FRANKLIN E.W., MC MILLAN I., 1979 - *The starch iodine test for determining stage of maturation in apples*. - Can. J. Plant Sci., 59: 725-735.

TAITI C., COLZI I., AZZARELLO E., MANCUSO S., 2017 a - *Discovering a volatile organic compound fingerprinting of* Pouteria lucuma *fruits*. - Fruits, 72(3): 131-138.

TAITI C., COSTA C., MENESATTI P., CAPARROTTA S., BAZIHIZINA N., AZZARELLO E., PETRUCCI A.W., MASI E., GIORDANI E., 2015 - *Use of volatile organic compounds and physicochemical parameters for monitoring the post-harvest ripening of imported tropical fruits*. - European Food Res. Technol., 241(1): 91-102.

TAITI C., MARONE E., LANZA M., AZZARELLO E., MASI E., PANDOLFI C., GIORDANI E., MANCUSO S., 2017 b - *Nashi or Williams pear fruits? Use of volatile organic compounds, physicochemical parameters, and sensory evaluation to understand the consumer's preference.* - European Food Res. Technol., 243(11): 1917-1931.

TATEO F., BONONI M., 2010 - *Headspace-SPME analysis of volatiles from quince whole fruits*. - J. Essential Oil Res., 22(5): 416-418.

TSUNEYA T., ISHIHARA M., SHIOTA H., SHIGA M., 1983 - *Volatile components of quince fruit (*Cydonia oblonga *Mill.)*. - Agric. Biol. Chem., 47(11): 2495-2502.

UMANO K., SHOJI A., HAGI Y., SHIBAMOTO T., 1986 - *Volatile constituents of peel of quince fruit,* Cydonia oblonga *Miller*. - J. Agric. Food Chem., 34(4), 593-596.

Adv. Hort. Sci., 2018 32(1): 113-135 DOI: 10.13128/ahs-21330

*Review paper*

AHS
Advances in Horticultural Science

# Bio-techniques for improvement of qualitative and quantitative traits in walnut (*Juglans regia*)

**U.N. Shah [1], J.I. Mir [2], N. Ahmed [3], A. Zaid [4], S. Jan [3], K.M. Fazili [1], S.H. Wani [5 (*)]**

1 *Department of Biotechnology, University of Kashmir, Srinagar, Jammu and Kashmir, India.*
2 *Central Institute of Temperate Horticulture (ICAR), Srinagar, Jammu and Kashmir, India.*
3 *Sher-i-Kashmir University of Agricultural Science and Technology, Jammu and Kashmir, India.*
4 *Plant Physiology and Biochemistry Laboratory, Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, 202002 Aligarh, India.*
5 *MRCFC, Khudwani, Sher-i-Kashmir University of Agricultural Science and Technology, Jammu and Kashmir, India.*

OPEN ACCESS

**(*) Corresponding author:**
shabirhussainwani@gmail.com

**Citation:**
SHAH U.N., MIR J.I., AHMED N., ZAID A., JAN S., FAZILI K.M., WANI S.H., 2018 - *Bio-techniques for improvement of qualitative and quantitative traits in walnut (*Juglans regia*).* - Adv. Hort. Sci., 32(1): 113-135



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 26 September 2017
Accepted for publication 27 October 2017

*Key words*: antioxidants, molecular markers, nutraceuticals, polyphenols, phytochemicals, walnut.

**Abstract: Walnut, *Juglans regia* (L.) is an economically significant plant for its immense nutritive and economic value. The breeding character of walnut has lent it a wide diversity in genetic characteristics. The principal vegetative and common traditional agronomic traits together with biochemical characterization i.e., karyotyping and isoenzyme expression have been the early research methods. However, these techniques are time-consuming and susceptible to the environmental variations. Literature is meager in the distribution, applied applications in general and the use of agriculture biotechnology in particular in case of walnut plants. The bio-techniques like molecular markers are adequate in number and there is little or no diversity in the method employed for research on walnuts. Despite basic research method, the organization of information, its retrieval and presentation structures, form elaboration experienced immense advancement via molecular markers such as RFLP, ISSR, RAPD AFLP, SSR and SNP. This appraisal in its first part provides detailed information regarding the present scenario of data on biogeographical distribution, health benefits of walnut worldwide and current applications in the agroforestry management, biochemical evaluations and applied uses of a walnut tree which is relevant for both basic and applied research. The review in its second part sheds light on the application of sophisticated agricultural biotechnology techniques such as use of molecular markers to evaluate, realize the full potential of walnut for increasing its quality, quantity and for its sustainable production which cannot be obtained through usual breeding techniques to meet the demands of a projected world population.**

## 1. Origin and history of walnut (*Juglans regia* L.)

Walnut *Juglans regia* (L.) is commonly recognized as "Jupiter's royal acorn". English walnut is commonly known as "Persian walnut" as it originated from ancient Persia and was adorned as for royalty. *Juglans regia* (L.) species is extensively cultivated in the temperate zones of the world for its best quality nuts and procurement of commercial timber (McGranahan and Leslie, 1991, 2009). Lateral fruit bearing walnut exhibit more genetic variation particularly among familial varieties. In ancient times, the recognition of walnut was spread vigorously through caravans via sea trade. Greek and Romans have lately acquired trend towards walnut cultivation to avert the speckled walnut growing along the coastal areas.

## 2. Botanical description of walnut

*Plant morphology*

*Juglans regia* (L.) is a deciduous perennial tree belonging to the family *Juglandaceae* and grown chiefly for its edible seeds. Walnut tree is predominantly an extensive canopy-dweller decurrent tree with trunk size ranging from 1.5-2 meters in diameter, 25-35 m in height and its survival rate is about 200 years. Walnut bears smooth bark with olive brownish color adapted to moderate light conditions. Leaves are 30-40 cm long, compound, alternately arranged and odd-pinnate. Male flowers are produced in drooping catkins and the female flowers, in clusters of two to five, are terminal, which ripens in the autumn into a fruit with a semi-fleshy husk and a brown nut. The kernel of the nut is protected by a corrugated woody shell.

*Flowering, pollination and fruiting*

*Juglans* are monoecious species, having male and female reproductive organs on separate flowers on the same tree. *Juglans regia* is self-fertile, heterogamous and it can be protandrous or protogynous depending on cultivar. The characteristic inflorescence is catkin bearing about 2-3 pistillate flowers born terminally or laterally. Walnut is self-fertile however occasionally it requires different cultivar due to dichogamy. Majority of walnuts are protandrous and anemophilous. Nuts are borne individually or in groups enumerating 2-3 on shoot tips. Walnut is properly shielded by a fleshy covering which cracks at maturity. Walnut shape is variable from ovoid to round attaining a maximum diameter of 2 meters enclosing two kernels divided by extremely delicate covering expanding from inner cover of the shell.

*Taxonomical classification*

Walnut is a member of *Juglandaceae* family and includes approximately 60 species, among which 21 species are classified under genus *Juglans* (Table 1). Different varieties of walnut and their commercial uses are given in Table 2. All walnut species are edible and English walnut is easily crackable and enormous in size.

Table 1 - Taxonomic classification of walnut

| Kingdom | Plantae |
|---|---|
| Phylum | Tracheophyta |
| Class | Magnoliopsida |
| Order | Fagales |
| Family | Juglandaceae |
| Subfamily | Juglandoideae |
| Tribe | Juglandeae |
| Subtribe | Juglandinae |
| Genus | *Juglans* |
| Species | ● *J. regia* Linnaeus |
| | ● *Juglans ailanthifolia* Carr. |
| | ● *Juglans ailantifolia* Carriere |
| | ● *Juglans bixbyi* Rehd. |
| | ● *Juglans boliviana* (C. DC.) Dode |
| | ● *Juglans californica* S. Wats. |
| | ● *Juglans cinerea* Linnaeus |
| | ● *Juglans hindsii* (Jepson) Jepson ex R. E. Sm. |

*Different sections and species of walnut*

Genus *Juglans* is classified into four types: *Juglans sect. Cardiocaryon, Juglans sect. Juglans, Juglans sect. Rhysocaryon,* and *Juglans* sect. *Trachycaryon.*

*Juglans sect. Cardiocaryon.* This variety originated from the northeast Asia. This walnut variety bears enormous leaves containing 15 leaflets. The wood is commercially soft, and nuts are thick shelled borne in a raceme inflorescence, such as, for example, *J. mandshurica* and *J. ailantifolia*.

*Juglans sect. Juglans.* Originated from the southeast Europe to central Asia. This variety bears large leaves with 9 broad hairless leaflets having entire margins. The wood is commercially hard, for example, *J. regia* (L.) and *J. sigillata.*

*Juglans sect. Rhysocaryon. (black walnuts)* Originated from the North America and the South America. This variety bears enormous leaves with 20

Table 2 - Different varieties of walnut and their commercial uses

| No. | Species | Description | Commercial uses |
|---|---|---|---|
| 1 | English/Persian Walnut (*Juglans regia*) | Native to the region of California, Oregon, Himalayas, Europe and China. Most ubiquitous cultivated in foothills having high water table. Characterized by a relatively thin, gnarled shell enclosing a smooth, large, ivory-colored nut and rich flavor. | As an effective nutraceutical with a high nutritive value. Procurement of high quality timber in the furniture industry for high-end flooring, guitars, furniture, veneers, knobs and handles as well as gunstocks |
| 2 | Eastern Black Walnut (*Juglans nigra*) | Native to eastern North America. Characterized by a thick, hard shell with sharp, jagged edges and a darker color. Black nuts are known for their pungent aroma and robust flavor. | Used in timber industry as well as nut production. The hard-black walnut shell is also used commercially in abrasive cleaning, a filtering agent in scrubbers in smoke stacks, cleaning jet engines, cosmetics, and oil well drilling and water filtration |
| 3 | White/ Butternut (*Juglans cinerea*) | Native to the eastern United States and southeast Canada. Characterized by slow-growing species, and rarely lives longer than 75 years. Bark is light grey in colour and the whole leaf is downy-pubescent, and a somewhat brighter, yellower green than many other tree leaves. Leaves are alternate and compound and have odd number of leaflets with a terminal leaflet | Used in lumber industry to make furniture, and is a favorite of woodcarvers. It is used to dye fabrics. Used as a medicine owing to its cathartic properties |
| 4 | Japanese Walnut/ Heartnut *(J. ailantifolia)* | Native to Japan and Sakhalin. Characterized by light grey bark, pinnate and brighter yellower green leaves, nuts produced in bunches of 4-10 and are of spherical shape | The edible nuts have an oily texture. The husks are also used to make a yellowish dye |
| 5 | Manchurian/ Chinese Walnut *(J.mandshurica)* | Native to the eastern Asiatic region (China, Russia and Korea). Characterized by odd, alternate, pinnate, long and broad leaves. Shell is thick and the kernels are edible but small and difficult to extract. Also contain less quantities of allelopathic compounds like Juglone | The timber is in use but is less valuable as compared to English or Black walnut. Cultivated as an ornamental in colder temperate regions |
| 6 | Iron Walnut (*Juglans sigillata)* | Native to the Yunnan, Guizhou, Sichuan and Xizang in China. Characterized by oval-shaped nuts with bumps and ridges. Cultivated for its edible nuts | Used as ornamental plant in gardens and parks |
| 7 | California Walnut (*Juglans californica)* | Endemic to California and generally found in southern California Coast Ranges, Transverse Ranges, and Peninsular Ranges, and the Central Valley. Characterized by a large shrub or a small tree. It has a small hard nut in a shallowly grooved, thick shell that is difficult to remove. It has a better flavour than *Juglans nigra* | Raw as well as cooked seeds are used in pies, cakes, biscuits, confections etc. An attractive wood, but the frequent branching pattern of the trunk limits the use of this wood commercially. Also has a medicinal value |
| 8 | Brazilian/ Argentine walnut (*J. australis*) | Native to Argentina and Bolivia. Characterized by dense, hard and strong wood. Its more frost resistant than *J. regia* | Nutritive value. The immature fruits are pickled whole for human consumption. The mature nuts are also eaten. The concentrated extract of the husk is also used as a vermifuge |
| 9 | Northern California walnut (*Juglans hindsii)* | Endemic to Northern California. Characterized by smooth, brown, thick shell, that contains a small edible nutmeat | Commercially important as a rootstock for orchard stock of *Juglans regia*. Also used as an ornamental tree in wildlife gardens, and for habitat gardens, natural landscaping projects. Its wood commonly called claro walnut is used in the lumber industry |
| 10 | West Indian walnut (West Indian walnut) | Not native to Jamaica but found in Cuba, the Dominican Republic, Haiti, and Puerto Rico. Characterized by a drupe 2-3 cms long with a black husk and a seed, which is an edible walnut meat, inside. It is listed as an endangered species in the Endangered Species Act of the United States | Used in timber industry. The attractive wood is similar to that of the black walnut |
| 11 | Cedro negro *(Juglans olanchana)* | Native to Costa Rica, Guatemala, El Salvador, Honduras, Mexico and Nicaragua. Commonly called cedro negro. Characterized by long branches bearing twigs tipped with 40-50 cm long, glabrous, pinnately compound leaves, darker on the top than on the bottom. The base of the trunk sometimes has buttresses | Used in the timber industry for light construction, cabinet-making, parquet floors, luxurious furniture, turnery, musical instruments, and veneer. The husk is used to dye leather |
| 12 | Colombian walnut (*Juglans neotropica)* | Native to Colombia, Ecuador, and Peru. Characterized by slow-growing tree up to 40 mts height with grooved, red-brown bark and an oval-shaped canopy. Leaves are compound with a serrated border | Nuts are used as food. Used in timber industry because the hard, durable wood is highly prized in cabinetry, flooring, veneers, utensils, and other forms of decoration |

leaflets, serrate margins. The wood is commercially very hard, such as, *J. californica, J. hindsii, J. australis, J. nigra,* and *J. olanchana.*

*Juglans* sect. *Trachycaryon.* Originated from the Eastern North America. This variety bears wide leaves containing 20 leaflets having serrate margins. The wood is commercially soft and the fruits are borne in clusters of two to three. The nuts have a thick and rough shell such as *J. cinerea* (L.).

## 3. World walnut production

Walnut grows abundantly in temperate areas of the world and cultivated commercially in 48 countries on an area of 1.6 million acres. The worldwide production of walnuts has reached maximum particularly in countries of Asia. Global walnut production for the year 2015-16 rose 155,000 tons from the previous year to 2.1 million tons, with China and the United States accounting for over 75 percent of total production (USDA, 2016). China is the world's chief producer of walnut and accounts for 51.49% of world's walnut production followed by the USA (26.86%), European Union (6.02%), Ukraine, Chile, Turkey and India contribute 5.25%, 3.09%, 3.09%, 1.96%, respectively to the world's walnut production (USDA, 2016). Global walnut production for the year 2016-17 is nearly unchanged from the October 2016 record forecast of 2.1 million tons in-shell basis, with China and the United States accounting for nearly 80 percent of total production (USDA, 2016). Figure 1 gives the schematic representation of year wise production of walnuts across the world.

## 4. Status of walnut in India

Walnuts are commonly called "Akhrot" in India and they are grown in the northwestern Himalayan belt, expanding up to Sikkim and Darjeeling. But the commercial farming of walnut is limited to the states of Arunachal Pradesh, Himachal Pradesh, Uttarakhand and Jammu and Kashmir. The area and production of walnut in India for the year 2014-15 were recorded to be 125,000 ha and 206,000 tons respectively (Government of India, 2015). India has exported 3,291.71 MT of walnuts worth of Rupees. 117.92 crores during the year 2015-16 (APEDA, 2016). Figure 2 gives the schematic representation year wise area versus production of walnut in India.

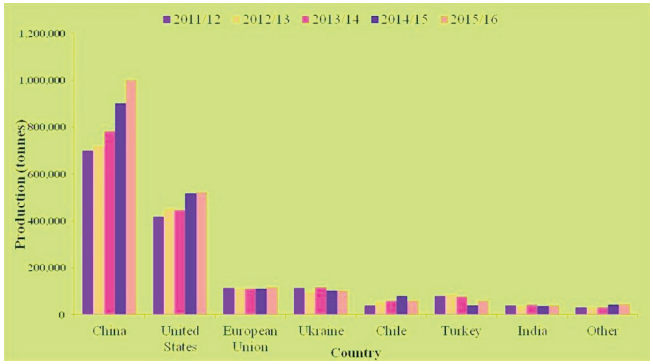



Fig. 1 - Schematic representation of walnut year wise production across the world.

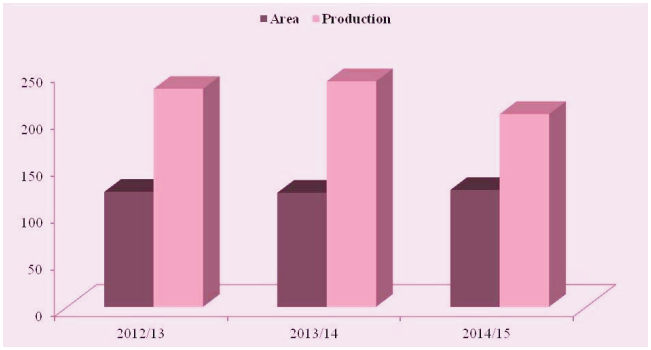



Fig. 2 - Shows the schematic representation of walnut production during three consecutive years in India.

The major walnut importing countries from India are Vietnam Social Republic (15%) followed by Egypt Arab Republic (11%), Netherland (11%), United Kingdom (9%), Spain (8%), United States (7%), Germany (6%), France (4%), Thailand (3%), Australia (3%) and others (23%) (Ministry of Agriculture, 2015) (Fig. 3).

Jammu and Kashmir is the main center for commercial walnut production in India and contributes pretty nearly 98% of the country's output and the


Fig. 3 - Showing principal countries which imports walnuts from India.

state has been declared as the "Agri. Export zone for Walnuts" (Isher *et al.*, 2016). Most walnuts that are produced for export by India are principally produced in this state. According to the United Nations Development Programme Special Unit for South-South Cooperation (UNDP SU/SSC), 63,000 hectares of Jammu and Kashmir state is under walnut cultivation. These plantings produce around 60,000 tones of walnuts worth an estimated 25 million Rupees.

## 5. Walnut as phytochemical

Walnut is popularly known for its abundant phytochemicals for instance phenolics, fatty acids, melatonin and serotonin (Christopoulos and Tsantili, 2015). In addition to kernels, green shells, walnut husk, seeds, bark and leaves are utilized in the cosmetic and pharmaceutical sector (Negi *et al.*, 2011; Fernández-Agulló *et al.,* 2013; Vinson and Cai, 2012). Table 3 gives a description of walnut and its phytoconstituents in diverse walnut varieties. The level of phenolics in walnut depends on diverse ecological factors, genotype, type of cultivar, geographical site, climatic parameters and developmental stages (Solar *et al.*, 2006; Amaral *et al.*, 2008). Toivonen and Hodges (2011) illustrated tissue and age specific variation in phenolic acids. Walnut polyphenols are potential candidates which work against *in vitro* plasma and low-density lipoprotein LDL oxidation. Regueiro *et al.* (2014) reported several individual phenolic compounds in walnut kernels and characterized approximately thirty-seven compounds. Colaric *et al.* (2005) demonstrated a greater amount

Table 3 - Description of walnut and its phytoconstituents in diverse walnut varieties

| No. | Walnut name | Part used | Phytochemical extracted | Reference |
|---|---|---|---|---|
| 1 | Persian walnut | Kernels | Phenolic acids (gallic acid, ellagic acid, syringic acid, chlorogenic acid, p-coumaric acid) and flavanols (catechin, epicatechin, gallocatechin, procyanidin B2, epigallocatechin, epicatechin gallate) | Figueroa *et al.,* 2016 |
| 2 | Persian walnut | Kernels | Ellagic acid | Fukuda *et al.,* 2004; Christopoulos and Tsantili, 2012 |
| 3 | Heartnut *Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.) | Kernels | Ellagic acid | Li *et al.*, 2006 |
| 4 | Persian Walnut | Leaves | Phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, p-coumaric, ferulic, sinapic, salycilic, ellagic and trans-cinnamic) flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone | Nour *et al.*, 2012 |
| 5 | Persian Walnut | Bark | Gallic acid, chlorogenic acid, catechine hydrate;resorcinol; caffeic acid, rutin trihydrate, syringic acid, vanillicacid,p-coumaric acid,quercitine dihydrate, naphto-resorcinol, trans-cinnamic acid etc | Noumi *et al.*, 2012 |
| 6 | Persian Walnut | Fruit | Phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, p-coumaric, ferulic, sinapic, salicylic, ellagic and trans-cinnamic acid) flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone. | Cosmulescu *et al.*, 2014 |
| 7 | Persian Walnut | Walnut husks | Chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone. | Stampar *et al.* 2006 |
| 8 | Persian Walnut | Kernels and pellicle | Chlorogenic, caffeic, p-coumaric, ferulic, sinapic, ellagic, and syringic acid. Syringaldehyde and juglone | Colaric *et al.*, 2005 |
| 9 | Persian Walnut | Leaves and husks | Chlorogenic acid, ferulic acid, gallic acid and trans-cinnamic acid | Chrzanowski *et al.*, 2011 |
| 10 | Persian Walnut | Leaves | Juglone | Thakur, 2011; Babula *et al.*, 2006 |
| 11 | Persian Walnut | Axillary shoots (microshoots) | Phenolic acids (gallaicacid, chlorogenic acid, p-coumaric acid, syringic acid, vanillin) and flavanoids (quercetin, naphthoquinone) and juglone | Cheniany *et al.*, 2013 |
| 12 | Persian Walnut | Fruit | Gallic acid, chlorogenic acid, sinapic acid, protocatechuic acid, catechin and juglone | Jakopič *et al.*, 2009 |

of phenolics in walnut skin than in kernels. Oliveira *et al.* (2008) confirmed protective properties of walnut husk exhibiting higher anti-microbial activity. Walnut kernels are known to be an immense resource of ellagitannins having higher anti-oxidant property (Shimoda *et al.*, 2009). Amaral *et al.* (2008) confirmed that presence of 96% of tannins as major causal agent for the medicinal potential of walnuts along with phenolics and flavanoids. Phenolic compounds are known to confer health benefits**.**

*Juglans regia* L. leaves are good sources of flavonoids. Many types of research are mainly focused on the extraction/isolation and the antioxidant effect of flavonoids. Walnut flavonoids have not been extensively analyzed, however, English walnut leaf was subjected to vigorous chromatographic evaluation (Pereira *et al.*, 2007). Amaral *et al.* (2008) and Pereira *et al.* (2007) analysed quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified quercetin 3-pentoside and kaempferol 3-pentoside derivatives in walnut leaves along with myricetin as exclusive flavonoid in walnut husk (Lugasi *et al.*, 2003; Stampar *et al.*, 2006). Myricetin exhibit exceptionally high anti-oxidant property along with the potential of imparting beneficial effects on bone heath thereby preventing bone resorption and elevating osteoblastic potential and bone formation (Hsu *et al.*, 2007). Rutin (Quercetin-3-rutinoside) is another significant flavonoid in walnut leaves having elevated levels of anti-oxidant potential that prevent DNA oxidation (Nour *et al.*, 2012).

The previous studies on flavonoids present in *Juglans regia* (L.) leaves provided a conjectural source for an additional study on the organic decoction from *J. regia* leaves. The promising results from the medicinal point of view were procured from flavonoids. Estimation and characterization of phenolic compounds in walnut leaves can be utilized as an important resource. Carvalho *et al.* (2010) reported phenolics and associated anti-oxidant characteristics of methanolic and petroleum ether extracts procured from walnut green shells and leaves. Seeds methanolic extracts exhibited highest phenolic content approximately 116 mg gallic acid equivalent (GAE)/g of extract with EC50 of 0.143 mg/mL using DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity followed by leaf and green husk. Solvent polarity was directly correlated with the distinct disparity in the activity of samples as observed by the solubility of phenolics compounds in respective solvents. Hence, methanol solvent is preferred for phenolics extraction (Do *et al.*, 2014) while petroleum ether is non-polar normally employed for extraction of lesser polar constituents for instance plant pigments, sterols and fatty acids (Carvalho *et al.,* 2010). Carvalho *et al.* (2010) reported maximum phenolics fraction (116.22±3.76 mg of GAE/g of seed extract followed by leaf 94.39±5.63 mg of GAE/g of extract) and green husk (50.18±2.69 mg of GAE/g of extract). Previous research reports have conformed methanolic extracts as most efficient in radical scavenging activity. Al-Okbi *et al.* (2014) reported phenolics content of 47.42 g GAE/100 g in ethanolic extract of walnut seed. Jalili and Sadeghzade (2012) illustrated the average value of 22.16±0.66 (mg/g) of phenolic content in aqueous extracts in diverse walnut cultivars. Ghasemi *et al.* (2011) illustrated the phenolic content of 15.15 to 108.11 mg gallic acid equivalents $g^{-1}$ of extract and flavonoid content ranging from 3.59 to 22.91 mg quercetin equivalents (QE)$g^{-1}$ of extract. Rahimipanah *et al.* (2010) reported total flavonoids and phenolics in walnut green husks via aluminum nitrate and Folin-Ciocalteu colorimetric protocol and their content were 1.44±0.2 mg quercetin and 34.28±1.35mg gallic acid equivalent per gram of dry sample correspondingly. Kale *et al.* (2010) demonstrated flavonoid fraction of walnut bark extracts with maximum quercetin between 3.50 to 32.81 mg/g and phenolics content varied from 20.32 to 44.87 mg/g in the extract. Ethyl acetate extract of walnut showing maximum phenolics and flavonoid sustaining antioxidant potential walnut extracts and recommend their utilization for advanced research to identify secondary metabolites.

## 6. Phytopharmacology of walnut

All the parts of walnut i.e., bark, leaves, flowers are exclusively used widely in Ayurveda, Unani, homeopathy and allopathic medicine system (Shah *et al.*, 2014). Forino *et al.*, 2016 reported conventional uses of walnut in health maintenance. Diverse activities of walnut utilized are due to enormous phytoconstituents such as saponins, glycosides, alkaloids, and steroids (Muthaiyah *et al.*, 2011). All parts of walnuts particularly leave and husk is extensively used in the pharmaceutical and cosmetic industries (Ribeiro *et al.*, 2015). Table 4 gives the description of walnut as phytochemical and its activity against diverse pathogens.

Table 4 - Description of walnut as phytochemical and its activity against diverse pathogens

| Walnut | Part used | Target cell/ pathogen | Activity | Reference |
|---|---|---|---|---|
| *Juglans regia* | Bark | *Staphylococcus aureus, E. coli* | Antibacterial | Farooqui *et al.*, 2015 |
| *Juglans regia* | Bark | *Candida albicans, Candida glabrata*, and *Candida parapsilosis* strains | Antifungal | Noumi *et al.*, 2010 |
| *Juglans regia* | Kernels | MCF-7 (estrogen receptor positive breast adenocarcinoma), KB (oral and mouth), HepG-2 (liver), Caco2 (colon), and WRL-68 (liver) | Anti-proliferative | Negi *et al.*, 2011 |
| *Juglans regia* | Leaves | Tobacco mosaic virus | Antiviral | Zhai *et al.*, 2007 |
| *Juglans regia* | Leaves | *Sindbis virus herpessimplex* (HSV), *Sindbis* (SINV), and *Poliovirus* | Antiviral | Mouhajir *et al.*, 2001 |
| *Juglans regia* | Leaves | *769-P renal* and *Caco-2 colon* cancer cells | Anti-proliferative | Carvalho *et al.*, 2010 |
| *Juglans regia* | Leaves | A375 human melanoma cell | Anti-proliferative | Shah *et al.*, 2015 |
| *Juglans regia* | Kernels | COLO-205 | Anti-proliferative | Anjum *et al.*, 2016 |
| *Juglans regia* | Walnut oil | *Herpes simplex* (HSV), *Parainfluenza* (PI-3) *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Enterococcus faecalis,* Fungi*: Candida albicans and Candida parapsilosis* | Antiviral, Antibacterial, Antifungal | Orhan *et al.*, 2011 |
| *Juglans regia* | Leaves | *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, Fungi: *Candida albicans, Cryptococcus neoformans* | Antibacterial and antifungal | Pereira *et al.*, 2007 |
| *Juglans regia* | Husks | *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, Fungi: *Candida albicans*, *Cryptococcus neoformans* | Antibacterial and antifungal | Oliveira *et al*., 2008 |

*Antimicrobial activity*

Antimicrobial potential of walnut especially bark (Farooqui *et al.*, 2015) leaves (Pereira *et al.*, 2007) and fruits (Pereira *et al.*, 2008) have been extensively utilized against various infections.

Studies have also demonstrated the antimicrobial activity of walnut products, particularly of bark, leaves, fruits and green husk of *Juglan regia* (L.) was subjected to hot and cold extraction and resulting solution exhibited high antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus epidermidis, Micrococcus luteus, Salmonella typhimurium, Enterococcus faecalis, Bacillus thuringiensis, Protomonas extroquens,* and Proteus sp., as evaluated through agar streak and disc diffusion assays (Biyik, 2010; Deshpande *et al.*, 2011).

*Antifungal activity*

Aqueous extracts of leaves and bark display antifungal activity against an extensive spectrum of fungi using disc diffusion, agar dilution, agar streak dilution and reddish assays. Pereira *et al.* (2008) demonstrated the enormous anti-fungal potential of walnut extracts refluxed with petroleum ether (b.p. 40-60°C) against *Candida albicans* and *Cryptococcus neoformans.* Noumi *et al.* (2010) demonstrated antifungal properties of *Juglans regia* (L.) alongside oral *Candida* strains.

*Antiviral activity*

Zhai *et al.* (2007) confirmed inhibition of tobacco mosaic virus (TMV) by 95% ethanol and ethyl acetate leaves extract of *J. regia*. At a minimum concentration of 1.5 µg/ml of methanolic extract of *J. regia,* there was distinct inhibition Sindhis (Mouhajir *et al.*, 2001).

*Antioxidant activity*

Several research reports demonstrated antioxidant activity of ethyl acetate, butanol, ether and aqueous methanol extract of walnut kernels, husks and leaves as evaluated by DPPH radical scavenging assay and lipid oxidation inhibition by β-carotene linoleate (Oliveira *et al.*, 2008; Pereira *et al.*, 2008; Carvalho *et al.*, 2010; Rahimipanah *et al.*, 2010;

Qamar and Sultana, 2011).

Consumption of walnuts and walnut skin diet by C57BL/6 mice showed decline oxidative stress as observed by a change in the enzymatic and non-enzymatic system (Bulló *et al.*, 2010). Furthermore, research report demonstrated ameliorating effect of walnut consumption on LDL oxidizing potential owing to their elevated levels of omega-6 PUFAs.

*Antidiabetic activity*

In Mediterranean and Asiatic countries, air-dried leaves of *Juglans regia* have been employed to treat diabetic symptoms (Hosseini *et al.*, 2014), and the potential of walnut leaves to ameliorate hyperglycemia in humans has been evaluated by both *in vitro* and *in vivo*. Pitschmann *et al.* (2014) reported inhibition of protein-tyrosine phosphatase 1B and no effect on peroxisome proliferator-activated receptors-gamma (PPARγ) in myocytes fed with methanolic extracts from walnut leaves. This study confirmed walnut leaf extracts drastically decline levels of serum fasting HbA1c, blood glucose and insulin (Hosseini *et al.*, 2014). Hence the study concluded hypoglycemic potential of walnut leaves wielding lowering effect on sugar levels in the liver as well as kidney. The mechanisms of action underlying the antihyperglycaemic effect of the walnut leaf extracts were investigated by *in vitro* studies. Inhibition of significant enzymes involved in sugar metabolisms like α-amylase (Rahimzadeh *et al.*, 2014), PTP1B (Ahmad *et al.*, 2012) and associated processes like glycation and oxidation reactions have been reported with walnut leaves (Pitschmann *et al.*, 2014).

*Antihelmintic activity*

A wide range of anti-worm activities was also reported from bark extracts of walnut. Kale *et al.* (2011) reported antihelminthic activity against *Eiciniafeotida* using reference Albendazole. Methanolic, ethanolic and benzene extracts of *Juglans regia* (L.) exhibited inhibitory activity against annelids like *Pheretimaposthuma* using standard drug Piperazine citrate (Upadhyay *et al.*, 2010).

*Anti-inflammatory activity*

Anti-inflammatory potential of walnut is comparatively inadequate. Papoutsi *et al.* (2008) demonstrated anti-inflammatory activity because of ellagic acid.

*Hepatoprotective activity*

Ameliorative effect of walnut polyphenols from kernel pellicle against a decline in glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) was reported in mice model after a single oral administration (200 g/kg) (Shimoda *et al.*, 2008). These results confirmed higher values of a hepatoprotective effect than curcumin.

## 7. Walnut and cancer

Due to the increasing inclination towards plant based drugs, more vigorous initiatives have been taken to action to develop more advanced plant based drug formulations. Among the diverse phytochemicals procured from plants, polyphenols are known to be most active candidates against cancer (Kim *et al.*, 2016). Singh and Sukhla (2015) demonstrated polyphenols as potent cancer inhibiting phytoconstituents as revealed by inhibitory effects of walnut on cancer promotion and progression. Polyphenols inhibit cancer progression by hampering nuclear factorkappa B - (NF-kB) activation. NF-kB is a nuclear transcription factor exhibiting significant role in gene regulation implicated in inflammation and carcinogenesis. Upregulation of NF-kB is implicated in cell-cycle progression. Polyphenol suppresses NF-kB and activator protein-1 (AP-1), its inhibition is considered as a potential step in chemoprevention. Polyphenols further induce inhibition in mitogen activated protein kinases (MAPK), protein kinases (PK), of the growth-factor receptor (GFR) leading to cell cycle arrest, the onset of apoptosis and decline in angiogenesis (Bonfili *et al.*, 2008). Mertens-Talcott *et al.* (2005) illustrated on the synergistic, additive, or antagonistic interface of polyphenolic compounds to counteract cancer growth. Shah *et al.* (2015) reported numerous chemical constituents possessing anticancer activity due to omega-3 fatty acids (Gerber, 2012), vitamin E (mainly the c-tocopherol form) (Albanes *et al.*, 2014), phytosterols (Ramprasath and Awad, 2015), ellagic acid (Eskandari *et al.*, 2016), gallic acid (Ho *et al.*, 2013) and flavonoids namely quercetin (Firdous *et al.*, 2014), carotenoids (Virtamo *et al.*, 2014), and melatonin (Travis *et al.*, 2014). Hardman (2014) reported 80% inhibition of breast cancer in mice following walnut consumption. Due to the presence of omega-3 fatty acids along with phytosterols in walnut breast cancer was declined remarkably. Phytosterols bind with estrogen receptors and subsequently decline breast cancer. Consumption of walnut diet declined a breast tumour by about 60% in transgenic mouse. Hardman (2014) confirmed presence of numerous complex constituents in walnut exhibiting additive or synergistic effect on cancer suppression. Further presence of

γ-tocopherol in walnut exhibit anti-cancer activities against prostate, colon, renal and lungs as demonstrated by anti-proliferative and anti-angiogenic mechanisms (Rafieian-Kopaie and Nasri, 2015). Walnut inhibits inflammation by inhibiting endothelin (Ma *et al.*, 2010). Research on prostate cancer established higher levels of endothelin in men indicating the need for further research to decipher relation between walnut and prostate cancer. Reiter *et al.* (2013) investigated prostate cancer and a determined decline in tumor and LNCaP (Lymph Node Carcinoma of the Prostate) xenograft growth. Negi *et al.* (2011) determined anti-proliferative activity of chloroform and ethyl acetate fractions of walnut in human cancer cell lines such as MCF-7 (Michigan Cancer Foundation-7) (estrogen receptor positive breast adenocarcinoma), KB (oral and mouth), HepG-2 (liver), Caco2 (colon), and WRL-68 (liver) cancer cell lines. All the cell lines exhibited inhibition against kidney and colon cancer however for 769-P renal and Caco-2 colon cancer cells, walnut leaf revealed the elevated rate of anti-proliferative competence than walnut seeds and husk. The observations procured confirmed lack of relationship among total phenols and anti-proliferative activities signifying some class of compounds implicated in cancer inhibition (Carvalho *et al.*, 2010). This study was further validated by Tsoukas *et al.* (2015) who demonstrated a converse association between walnut intake and colon cancer incidence. Paur *et al.* (2010) reported dose dependent inhibition in lipopolysaccharide(LPS)-induced NF-κB activity in the monocytic cell line (U937-κB) by blending clove, oregano, thyme, walnut and coffee. This study confirmed the comparative efficacy of the combination of different extracts than individual plant constituent. Hence, walnut can be utilized as a natural source of anti-oxidants as well as chemo preventive candidate. Thakur (2011) demonstrated inhibition of carcinogenesis in rats by azoxymethane and can be utilized as chemo preventive agent against neoplasia. During recent years, juglone is established as effective cytotoxic agent and induce cell apoptosis via mitochondria dependent pathway via human lung cancer (A549) cells (Cenas *et al.*, 2006), human leukemia (HL-60) cells (Xu *et al.*, 2012 a), and human cervical carcinoma (HeLa) cells (Zhang *et al.*, 2012). Ji *et al.* (2008) reported inhibition of growth and induction of apoptosis of sarcoma 180 cells *in vivo.* Juglone, being an inhibitor of Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) is an impending remedial target for anti-cancer research. Role of Pin1 in relation to tamoxifen-resistant breast cancer has been an important determinant factor in drug resistance. These observations demonstrate Pin1 augment E2F-4 and Egr-1-driven expression of LC-3, an autophagosome marker is over expressed in cancer cells than non-cancerous. Namgoong *et al.* (2010) further established juglone as potent inhibitor having a distinct declining effect on the TPA and induced overexpression of E2F-4 and Egr-1 transcription factors controlling LC-3 gene expression.

## 8. Walnut as a nutraceutical

The term "nutraceutical" is used to illustrate food or food nutrient supplement that suggests a medical or health benefit by providing simple nutrition (Wong *et al.*, 2015). Functional foods are known to be a remedy for chronic diseases (Luciano, 2014). Walnut has been used as functional food. They are important components of the Mediterranean diet (Ley *et al.*, 2014). Nutritional profile of walnut is tabulated in Table 5. Not only the kernel of walnut but also green shells and bark have also been used in the cosmetic and pharmaceutical industries (Ribeiro *et al.*, 2015; Adeel *et al.*, 2017). Walnuts are chiefly consumed for their organoleptic and nutritional aspects since they are resources of important anti-oxidants and preserve food attributes (Martinez and Maestri, 2016). Walnut seeds are now established as major nutraceutical owing to their effects on coronary heart disease (Nasri *et al.*, 2015; Schwingshackl *et al.*, 2017). Walnuts possess numerous nutritional aspects including a maximum fraction of polyphenolics due to which they are incorporated into recommended dietary supply (Sanchez-Gonzalez *et al.*, 2017). Christopoulos and Tsantili (2015) reported numerous phytochemicals in walnut for example phenolics, fatty acids, melatonin and serotonin. In addition to phytochemicals, various hydrolyzable and condensed tannins have been reported in walnut (Figueroa *et al.*, 2016). High oil and protein content of kernel of *Juglans regia* (L.) (Juglandaceae) validates this fruit requisite for human nutrition. FAO has categorized walnut as a deliberate candidate for human nutrition and phytomedicine (Gandev, 2007). Most of the energy in walnuts generates from fats which account for 65% which makes them energy-rich and high-calorie food (Tapsell *et al.*, 2009). Martinez and Maestri (2008) reported triacylglycerols, in which monounsaturated fatty acids (MUFAs) mainly oleic acid (18:1 n-9) and polyunsaturated FAs (PUFAs;

Table 5 - Nutritional profile of Walnut

| Nutrients | Total amount /100 gm walnut |
|---|---|
| Calories | 654 |
| Water | 4% |
| Protein | 15.2 g |
| Carbs | 13.7 g |
| Sugar | 2.6 g |
| Sucrose | 2.4 g |
| Glucose | 0.1 g |
| Fructose | 0.1 g |
| Fiber | 6.7 g |
| Fats | 65.2 g |
| Saturated | 6.13 g |
| 16:00 | 4404 mg |
| 18:00 | 1659 mg |
| Monounsaturated fatty acids | 8.933 g |
| 18:01 | 8799 mg |
| 20:01 | 134 mg |
| Polyunsaturated fatty acids | 47.174 g |
| Omega-3 | 9.08 g |
| Omega-6 | 38.09 g |
| Trans fat | 0 |
| Vitamins | |
| Vitamin A | 1 µg |
| Vitamin C | 1.3 mg |
| Vitamin D | 0 µg |
| Vitamin E | 0.7 mg |
| Vitamin K | 2.7 µg |
| Vitamin B1 (Thiamine) | 0.34 mg |
| Vitamin B2 (Riboflavin) | 0.15 mg |
| Vitamin B3 (Niacin) | 1.13 mg |
| Vitamin B5 (Panthothenic acid) | 0.57 mg |
| Vitamin B6 (Pyridoxine) | 0.54 mg |
| Vitamin B12 | 0 µg |
| Folate | 98 µg |
| Choline | 39.2 mg |
| Minerals | |
| Calcium | 98 mg |
| Iron | 2.91 mg |
| Magnesium | 158 mg |
| Phosphorus | 346 mg |
| Potassium | 441 mg |
| Sodium | 2 mg |
| Zinc | 3.09 mg |
| Copper | 1.59 mg |
| Manganese | 3.41 mg |
| Selenium | 4.9 µg |
| Cholesterol | 0 mg |

linoleic (18:2 n-6) and α-linolenic acids (18:3 n-3) in walnut oil are present in high amounts in all genotypes. Walnuts are a chief resource for omega-6 fatty acid called linoleic acid. They also contain a relatively high percentage of omega-3 fat called alpha-linolenic acid (ALA) contributing up to 8-14% of the total fat content. Deckelbaum and Torrejon (2012) reported walnut as the sole source of ALA which declines inflammation and augments blood profile. Muradoglu *et al.* (2010) and Martinez *et al.* (2010) reported average protein estimation of about 18.1% comprising of 70% of total seed protein along with lesser amounts of globulins (18%), albumins (7%) and prolamins (5%). Walnut proteins possess all essential amino acids requisite for humans. Amino acids exist in proper ratio in walnuts e.g., lysine/arginine ratio in walnut proteins is comparatively lower than in daily vegetable proteins. The lower lysine/arginine ratio declines atherosclerosis development (Martinez *et al.*, 2010). In addition to macronutrients, walnuts are richest sources of vitamins and minerals, Potassium (K), phosphorus (P), magnesium (Mg) and iron (Fe) (Cosmulescu *et al.*, 2009). Calcium (Ca), sodium (Na), zinc (Zn) and Copper (Cu) are present in moderate fractions (Siahnouri *et al.*, 2013). Macro elements are necessary for proper development and growth of organisms (Jeszka-Skowron *et al.*, 2016). Most of macroelements assist in enzymatic reaction involved in the general metabolism of the organism. Trace elements promote heart health, maintains bone, nerve and immune system functions (Abdel-Aziz *et al.*, 2016). Vitamins are indispensable organic substances and in addition to their role in important metabolic functions, they are also required for proper functioning of hormone regulation. Anjum *et al.* (2016) confirmed presence of ellagic acid, gallic acid, tocopherol (vitamin E), ellagitannins (tannins) and phytochemicals with potential antioxidant activity, for instance, melatonin. Walnut also comprises of elevated levels of α- tocopherol, a vitamin E, which prevents lipid oxidation process and decline risk of cancer and coronary heart disease (Jiang, 2014).

*Health effects of walnut (Table 6)*

*Cardiovascular benefits.* Nijike *et al.* (2015) reported daily consumption of walnut lowers risk of heart diseases. Higher anti-oxidant and elevated levels of the fat fraction in walnut are important for heart health.

*Brain health.* Brain function is immensely advanced by eating nuts. Walnuts are also associated with alleviation of depression and age-linked diseases (Grosso and Estruch, 2016). Arab and Ang (2015) reported daily consumption of walnuts enhance memory retention.

*Walnuts help reduce problems in metabolic syndrome.* Metabolic syndrome is complex disorder encompassing increased triglycerides, high blood

pressure, insufficient high-density lipoproteins, cholesterol, and obesity. Saneei *et al.* (2013) reported daily consumption of one ounce of walnuts for 2-3 months can lower risk of MetS- associated disorders.

*Benefits in treatment of type 2 diabetes.* Type 2 diabetes is a growing problem in developed and industrialized countries. A better understanding of dietary changes is needed to improve this disorder. The significant diet regime for type 2 diabetes patients for lowering cardiovascular issues has been reported by consumption of walnuts on daily basis (Farr *et al.*, 2017).

*Anti-cancerous effects*. Walnuts contain a wide variety of antioxidant and anti-inflammatory bioactive components that may have anti-cancerous properties (Byerley *et al.*, 2017). Presence of polyphenolic compounds, phytosterols, gamma-tocopherol, omega-3 fatty acids and ellagic acid decline threat of chronic oxidative stress and ameliorate inflammatory properties to counteract intimidation of cancer advancement (Perugu and Vemula, 2014). Choi *et al.* (2016) and Al-Mahmood *et al.* (2016) reported a lower incidence of colon, breast and prostate cancer associated with walnut consumption.

*Other health benefits*. Walnut nutrients exhibit significant function in the maintenance of bone structure (Kajarabille *et al.*, 2013). It has been recently confirmed that walnut consumption decline blood levels of N-terminal telopeptideNTx of type 1 collagen. More collagen composition signifies higher bone stability as well as lower mineral loss from bones (Griel *et al.*, 2007). Katz *et al.* (2012) confirmed the potential significance of walnut consumption in body weight management.

## 9. Application of molecular markers in horticultural crops

Horticultural crops are important for dietary purpose and source of revenue for farmers in the emerging countries (Behera and France, 2016). Genetic

Table 6 - Health effects of walnut

| Cardiovascular aspect | Walnut benefit |
|---|---|
| Blood quality | Lowering LDL, the “bad” cholesterol; decreased total cholesterol; increased gamma-tocopherol; increased omega-3 fatty acids in red blood cells (alpha-linolenic acid) |
| Vasomotor tone | Decreased aortic endothelin; improved endothelial cell function |
| Risk of excessive clotting | Decreased maximum platelet aggregation rate; decreased platelet activation |
| Risk of excessive inflammation | Decreased C reactive protein (CRP); decreased tumor necrosis factor alpha (TNF-a) |
| Blood vessels | Improves the function of blood vessels by cutting the risk of plaque buildup in the arteries |
| Brain tonic | Relieves depression and age-related decline in brain function |
| Decline in metabolic syndrome | Reduction of various MetS-related problems |
| Treatment of type 2 diabetes | Regulates blood sugar and insulin metabolism |
| Development of body structure | Decrease blood levels of N-telopeptides of type 1 collagen (NTx); weight maintenance and prevention of obesity |
| Anti-cancer activities | Anti-inflammatory properties help lower risk of chronic inflammation |

improvement in horticultural crops is not yet as noteworthy as it has been achieved in case of cereals and grasses. The advancement and accessibility of genomic resources in these crops can be utilized resourcefully to conserve and investigate existing genetic diversity and to understand the association between genotype-phenotype and to accelerate breeding (Chaturvedi and Sahijram, 2015). Current progress in mechanization and high-throughput sequencing can be utilized to decipher ambiguous and intricate genomes. Gene pyramiding and polygenic resistance evaluation in diverse genotypes with extensive resistance have been made possible through the advent of molecular markers (Ansari, 2015). Walnut is valued for its wood and nut, phytochemicals but so far as its transcriptomics and genomic data is concerned, it is very limited (Hu *et al.*, 2016).

From the past few decades, a significant progress has been achieved by the use of molecular markers in plant biotechnology and unraveling their genomic intricacies for detecting and exploiting DNA polymorphism in the plants. There are different markers, i.e. markers based on the morphology of plants, on biochemical functions and DNA-based molecular markers (Parveen *et al.*, 2016). These molecular markers are classified as hybridization-based or non-PCR-based markers. By using, these DNA based molecular markers, one can get complete information about a particular plant from the level of the nucleotide to a segment on DNA and frequencies of alleles, population structure and distribution of genetic diversity. Molecular markers explicitly reveal the polymorphism at DNA level (Deoxyribonucleic acid). They are routinely used in various crop improvement programs, ecological, physiological, genetic studies of plants and to progress the effectiveness and accuracy of traditional plant breeding through marker assisted selection (Allwright and Taylor, 2016). Important information for genetic diversity can be evaluated for different horticultural crops by using RAPD markers, for example, eggplant and watermelon (Trivedi *et al.*, 2016), *Moringa oleifera* (L.) (Kumar *et al.*, 2017). AFLP markers have effectively been utilized for evaluating genetic diversity in horticultural crops like *Celosia argentea* (Olawuyi *et al.*, 2016), *Rosa platyacantha* (Yang *et al.*, 2016) and olive (Mnasri *et al.*, 2017). Owing to their hyper variability and competence in distinguishing of polymorphisms, SSR have become ideal markers for assessment of genetic diversity in horticultural crops like potato (Ghebreslassie *et al.*, 2016), grapes (Rao, 2017), high density genetic map construction (Xue *et al.*, 2008), population and conservation genetic studies, clonal identification (Liu *et al.*, 2016 a, b), controlled crosses certification, species and hybrid identification, paternity determination (De La Rosa *et al.*, 2013), marker assisted selection (Ashraf and Foolad, 2013) and genotyping (Billot *et al.*, 2013). ISSR is extensively utilized in population genetics of horticultural crops i.e., *Elaeis guineensis* (Chagas *et al.*, 2015) and *Rhododendron triflorum* (Xu *et al.*, 2017) and determining the genetic diversity of horticultural crops such as bael (*Aegle marmelos* Corr.) (Mujeeb *et al.*, 2017). This section of the chapter provides a detailed account of the exploitation of different molecular markers used in case of walnut study. Use of some like random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), single Nucleotide Polymorphism (SNP) are briefly discussed in improving, increasing the quality and quantity of walnut is summarized.

Single Nucleotide Polymorphism (SNPs) have been widely used in plant germplasm management and breeding of fruit tree crops (van Nocker and Gardiner, 2014). These benefits have resulted in SNPs gradually becoming the markers of choice for exact genotype identification and diversity analysis in horticultural crops such as cacao (*Theobroma cacao*) (Fang *et al.*, 2014 a), grapevine (*Vitis vinifera*) (Cabezas *et al.*, 2011), pummelo (*Citrus maxima*) (Wu *et al.*, 2014), strawberry (*Fragaria* spp.) (Longhi *et al.*, 2014) and tea (*Camellia sinensis*) (Fang *et al.*, 2014 b).

## 10. Molecular characterization in walnut

Molecular characterization of walnut germplasm is mandatory requirement for establishing breeding purposes and establishment of proprietary rights. Conventional approaches for germplasm characterization are evaluated based on comparative morphological analysis. Due to the ecological effects on phenotypic expression together with juvenile phase of walnut, there are ample hindrances in proper classification of walnut. To surmount these restrictions, molecular markers are employed for discrimination and identification of walnut accessions. These DNA based markers are not influenced by environment and can be identified in all development stages of plant tissues. Characterization of genetic variability in walnut was done using isozymes (Malvolti *et al.*, 1994), to identify interspecific hybrids (Arulsekar *et*

*al.*, 1985), in species and cultivars differentiation (Vyas *et al.*, 2003) and to evaluate mating aspects (Rink *et al.*, 1994). RFLP markers has been employed for evaluating parentage and ascertaining phylogenetic associations and discriminate among species and cultivars in the genus *Juglans* (Aly *et al.*, 1992; Fjellstrom *et al.*, 1994; Fjellstrom and Parfitt, 1995). Description of various molecular markers used in molecular characterization of various walnut genotypes is given in Table 7.

*Randomly amplified polymorphic DNA (RAPD)*

RAPD is immensely significant in deciphering closely associated cultivars in walnut and results obtained are harmonized with preexisting data procured from RFLPs owing to more vigorous polymorphism exhibited by RAPD (Nicese *et al.*, 1998). Furthermore, RAPD markers were also employed to assess polymorphism intensity at interspecific level among Persian walnut (*J. regia*) and in the Northern California black walnut [*J. hindsii* (Jeps.)] (Woeste *et al.*, 1996 a) and to recognize a marker associated with hypersensitivity to the cherry leaf-roll virus (Woeste *et al.*, 1996 b). Nicese *et al.* (1998) evaluated eighteen RAPD primers among nineteen walnut genotypes that comprised of strongly associated cultivars and parents in breeding programs. Following cluster analysis of closely associated genotypes, genotypes can be segregated into two classes having association with their closely related alleles. RAPD markers can investigate polymorphism to decipher variation among walnut genotypes particularly strongly associated genotypes and genetic similarity subsisting on RAPDs revealing can detect enough polymorphism to differentiate among walnut genotypes, even among closely identified (Hayward *et al.*, 2015). RAPD markers are highly useful for evaluating genetic variability in genus *Juglans* through DNA fingerprinting to differentiate between valuable genotypes for selection (Pop *et al.*, 2010; Ahmed *et al.*, 2012). RAPD was employed to evaluate association among eight walnut genotypes thriving in Turkey and discrimination among indigenous and exotic genotypes together with their early bearing progenies (Erturk and Dalkilic, 2011). Xu *et al.* (2012 b) illustrat-

Table 7 - Description of various molecular markers used in molecular characterisation of various walnut genotypes

| Walnut species/variety | Molecular marker used | Traits associated | References |
|---|---|---|---|
| Juglans regia | RFLP | Inheritance and linkage | Fjellstrom *et al.*, 1994 |
| *Juglans regia* | RAPD and SSR | Phenotypic | Pop *et al*., 2013 |
| *Juglans regia* | SSR | Morphological | Mahmoodi *et al*., 2013 |
| *Juglans regia* | SSR | Morphological | Karimi *et al.*, 2014 |
| Butternut (*Juglans cinerea* L.), Japanese Walnut (*Juglans ailantifolia*) and Buartnut *(Juglans cinerea* × *Juglans ailantifolia*) | SSR | Nut phenotype | Chen *et al.*, 2014 |
| *Juglans regia* | RAPD | Butternut canker disease resistance | Zhao *et al.*, 2014 |
| *Juglans nigra*, *Juglans regia*, and hybrid (*Juglans x intermedia* (Carr) | RAPD | Association between native/foreign genotypes with their early-bearing natural hybrids | Erturk and Dalkilic, 2011 |
| *Juglans regia* | SSR | Identification of interspecific hybrid | Pollegioni *et al.*, 2014 |
| *Juglans regia* | SSR | Morphological | Ebrahimi *et al.*, 2011 |
| *Juglans regia* | ISSR | Morphological and biochemical traits | Malvolti *et al.*, 2010 |
| *Juglans regia* | ISSR | Distinction between native and international cultivated genotypes | Christopoulos *et al.*, 2010 |
| *Juglans regia* | SLAF | Disease resistance to anthracnose | Zhu *et al.*, 2015. |
| *Juglans regia* | AFLP | Geographical proximity | Bayazit *et al.*, 2007 |
| *Juglans regia* | SNP | Fingerprinting of 30 walnut genotypes | Ciarmiello *et al.*, 2013 |
| *Juglans regia* | SNP | Genome sequencing | Liao *et al.*, 2014 |

ed RAPD and AFLP for deciphering genetic diversity of walnuts in western Sichuan plateau and Qinba mountainous regions. The walnut genotypes were procured from 8 different regions and 32 RAPD primers and 28 AFLP primers in combination were recognized with polymorphic bands in entire walnut. In this study, high allelic number and elevated genetic diversity identified with RAPD and AFLP signify western China has significant genetic diversity resource and profuse genetic variance among walnuts. Zhao *et al.* (2014) employed RAPD as chief molecular implement for characterizing genome of butternut (*Juglans cinerea*), Japanese walnut (*Juglans ailantifolia*), black walnut (*Juglans nigra*), Persian walnut (*Juglans regia*), Manchurian walnut (*Juglans mandshurica*), and an interspecific hybrid (*J. ailantifolia cinerea*) for species-specific markers.

The study revealed 38 amplicons were exclusive to Japanese walnut and buartnut hybrids lacking in butternut. RAPD markers were relatively insufficient to differentiate intraspecific variability inside Japanese walnut, butternut, or their hybrids. RAPD can also decipher hybrid lineage as in the case for discriminating whether Japanese walnut or Manchurian walnut was the progenitor of hybrid line or not. The results demonstrated that some of the hybrids can be distinguished through morphological and genetic resemblances through RAPD as it demonstrates pedigree data. The resulting RAPD markers provide their utility in the conservation of butternut, an endangered North American species.

*Simple sequence repeat (SSR)*

SSR is enumerated among most precise molecular marker owing to their high polymorphism, co-dominant transmission, higher reproducibility, exhibiting higher resolution and more convenient PCR detection in deciphering genetic relationship (Miah *et al.*. 2013). Wang *et al.* (2015), Ali *et al.* (2016), Ebrahimi *et al.* (2016), and Pang *et al.* (2017) illustrated research reports where in genetic characterization of walnut was evaluated using SSR. Han *et al.* (2016) described genetic diversity and population structure study of *J. regia* germplasm using ten primers developed from expressed sequence tags (EST-SSR) and sequence polymorphisms among the phenylalanine ammonia lyase (PAL) gene. The result showed high level of population differentiation. In addition, SSR markers informative in *Juglans regia* may also be polymorphic in other *Juglans* (L.) species (Aldrich *et al.*, 2003) and have utility for breeding hybrid rootstocks. SSR are more consistent and trustworthy system for molecular characterization. SSR has wide numerous relevance in case of walnut comprising of cultivar characterization, cultivar identification, pedigree substantiation for cultivar and in deciphering evaluation of superior rootstock for breeding and paternity analysis (Dangl *et al.*, 2005). Ruiz-Garcia *et al.* (2011) employed 32 SSR primer pairs to characterize 57 walnut cultivars originated from Spain and USA. In this research reports, 32 primer pairs flanking simple sequence repeats, were established in *Juglans nigra* to screen elite variety with high rate of polymorphism. Further selection of 19 selected microsatellite markers provided differentiation of evaluated cultivars exhibiting 97 alleles and on an average 5 alleles per locus establishing SSR as primary choice for characterization of walnut germplasm. The data generated from SSR characterization further confirmed molecular parameters of Spanish walnut to be different from Californian genotypes. Ahmed *et al.* (2012) illustrated genetic association of 82 walnut genotypes using 13 SSR and 20 RAPD primers exhibit higher level of genetic diversity in these walnut cultivars thriving rigorously in climate of Jammu and Kashmir. These results demonstrate applications of SSR markers in walnut breeding and conservation. Pollegioni *et al.* (2014) illustrated genetic diversity and spatial genetic structure of 39 autochthonous Persian walnut populations analyzed transversely along Asian range via 14 neutral microsatellite markers. Shah *et al.* (2016) reported genetic characterization of 96 walnut genotypes growing in North western Himalaya as investigated by 19 SSR markers and the study displayed high polymorphism rate of 89.6%. Chen *et al.* (2014) demonstrated potential of SSR markers in the cultivar characterization together with intellectual property rights. Topcu *et al.* (2015) developed genomic libraries augmented with CA, GA, AAC, and AAG repeats via genomic DNA from *J. regia* cv. Maraş-18 to develop SSR markers for walnut. This study confirmed GA-enriched library as preferred alternative in terms of allelic number, polymorphism, productivity, and information content.

*Inter simple sequence repeat (ISSR) markers*

ISSR-PCR is a most convenient approach that surmounts most of these restrictions encountered by other molecular markers (Karimi *et al.*, 2014). ISSR is most popular among plant improvement. Potter *et al.* (2002) has demonstrated the application of inter-simple sequence repeat (ISSR) markers in genetic characterization of English or Persian walnut (*Juglans regia* L.). In this study eight ISSR primers presented exclusive fingerprint for 48 cultivars investigated. The dendrogram developed from data presented classes

pertaining to known pedigrees which does not provide this data anticipating the fact that there is restriction in context to utility of ISSR in determining genetic association among species. Christopoulos *et al.* (2010) reported immense application of ISSR markers in genetic characterization for deciphering genetic diversity among Greek natives of walnut (*Juglans regia* L.). In this study, similarity coefficient values signified presence of a higher extent of genetic variability. Majority of international cultivars were classified together whilst majority of Greek native populations cannot be classified in separate class. International cultivars exhibit lesser diversity than Greek native population genotypes. The pairwise regional PhiPT values signified that most geographically isolated regions are the most genetically differentiated. The intense variability in Greek germplasm along with their desired traits anticipated that native germplasm can be utilized for breeding purposes and preservation of walnut germplasm. Li *et al.* (2011) employed ISSR markers to evaluate the genetic variation and genetic structure to provide a theoretical basis and technical support for appropriate conservation and application of existing genetic resources of walnut. Analysis of ANOVA (Analysis of variance) also showed genetic variance among populations was larger than within a population. Mantel test and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram based Nei's genetic distance displayed that genetic distance between populations had more significant correlation with geographic distance. Ji *et al.* (2014) developed ISSR primers to assess the degree and design of genetic diversity among eight populations of North China Mountain Walnut (NCMW). This diversity was further confirmed through ANOVA analysis. Structure of NCMW and UPGMA cluster analysis demonstrated restricted gene flow, habitat devastation and geographical isolation might be determining factors for population structure. UPGMA cluster further signify that eight populations of walnut can be classified into three discreet groups as per similarity coefficient and geographic origin but exhibited significant association with morphological traits especially nuts. Hu *et al.* (2016) in their classical experiments use transcriptomic information from RNA-Seq to understand development of polymorphic simple sequence repeats (SSRs, microsatellites) for understanding the population genetics of walnut. They studied more than 47.7 million clean reads, 99,869 unigenes having length of 747 bp. They further identified 39,708 (42.32%) genes, 63 new transcriptome-derived microsatellite markers. The identification and characterization of microsatellite markers in their study could help to explore the diversity, genetic structure, population genetics for improved walnut future breeding practices, besides providing a useful genetic resource information for studying and understanding the genomic and transcriptome aspect of walnut. Doğan *et al.* (2014) evaluated genetic association of 59 walnuts (*Juglans regia* L.) genotypes, international and Turkish by using three different types of molecular markers i.e. RAPD, ISSR and SSR primers. These results exhibited that SSR markers offer elevated rank of polymorphism as compared to RAPD and ISSR.

*Single nucleotide polymorphism (SNP) markers*

SNP is the most abundant type of DNA variation in most species. The introduction of next generation sequencing (NGS), together with high throughput genotyping technology, makes it relatively easy to identify and use SNPs (Elshire *et al.*, 2011). The new generation of molecular markers based on single nucleotide polymorphisms (SNP) represent a promising and effective tool for fast and accurate species identification. Ciarmiello *et al.* (2013) developed a simple amplification refractory mutation technique, based on SNP markers of rDNA and cox2 intron I sequences, to fingerprint 30 walnut genotypes. rDNA sequences revealed the presence of 402 variations and Cox2 intron I sequences showed 769 variable positions. The findings revealed that the cox2 intron I region, either alone or in conjunction with rDNA, could be used effectively in identifying these walnut genotypes. Liao *et al.* (2014) performed genome sequencing of walnut and then all the sequence reads were mapped against genome assembly of walnut. In total, 49,202 nucleotide variations were detected including 48,165 single nucleotide polymorphisms (SNP5) and 1037 insertions/deletions (InDels).

## 11. Conclusions

The nutritional factors of walnut are improved due to the presence of numerous micro elements. The notable nutritive feature of walnuts relates to a rich range of polyphenols. The breeding character of walnut has bestowed it an inclusive diversity in genetic features. These results have been attained after an extended progression of evolution and by going through intricate environmental abnormalities. However, these techniques are susceptible to the environmental variations. The markers are adequate

in number and there is little or no diversity in the method employed for research on walnuts. Despite basic research method, the organization of information, its retrieval and presentation structures, form elaboration experienced immense advancement via molecular markers such as RFLP, ISSR, RAPD AFLP, SSR and SNP. This assessment offers data regarding health benefits of walnut at the global level and existing applications in the horticulture. The present work comprehensively describes the utilization of molecular markers in walnut plants which could help to improve/enhance the traits linked to its optimum/yield growth and sustainable production of innumerable essential metabolites having the ability to find applications in horticulture and allied science (Fig. 4).

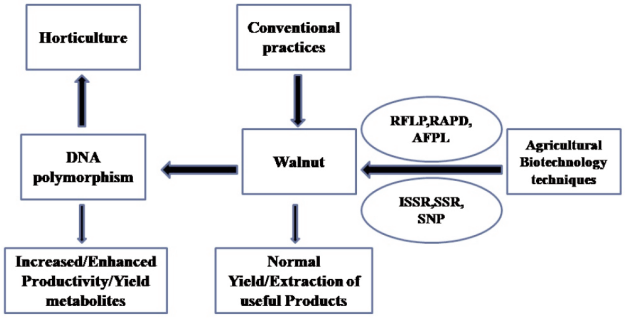



Fig. 4 - Schematic representation of utilization of molecular tools to improve walnut traits.

## Acknowledgements

The authors express their gratitude to Central Institute of Temperate Horticulture (Indian Council of Agricultural Research, (ICAR), Aligarh Muslim University and University Grants Commission New Delhi, India for financial support of this work.

## References

ABDEL-AZIZ S.M., AERON A., GARG N., 2016 - *Fortified foods and medicinal plants as immuno modulators*, pp. 143-162. - In: GARG N., S.M. ABDEL-AZIZ, and A. AERON (eds.) *Microbes in Food and Health*. Springer International Publishing, Cham (ZG) Szitzerland, pp. 362.

ADEEL S., RAFI S., SALMAN M., 2017 - *Potential resurgence of natural dyes in applied fields,* pp. 1-26. - In: UL-ISLAM S. (eds.) *Plant based natural products: derivatives and applications*. - Scrivener Publishing LLC. Beverly MA, USA, pp. 240.

AHMAD H., KHAN I., WAHID A., 2012 - *Antiglycation and antioxidation properties of* Juglans regia *and* Calendula officinalis*: possible role in reducing diabetic complications and slowing down ageing.* - J. Tradl. Chin. Med., 32(3): 411-414.

AHMED N., MIR J.I., MIR R.R., RATHER N.A., RASHID R., WANI S.H., SHEIKH M.A., 2012 - *SSR and RAPD analysis of genetic diversity in walnut (*Juglans regia *L.) genotypes from Jammu and Kashmir, India.* - Physiol. Mol. Biol. Plants, 18: 149-160.

ALBANES D., TILL C., KLEIN E.A., GOODMAN P.J., MONDUL A.M., WEINSTEIN S.J., FLESHNER N.E., 2014 - *Plasma tocopherols and risk of prostate cancer in the Selenium and Vitamin E Cancer Prevention Trial (SELECT)*. - Cancer Preven. Res., 7: 886-895.

ALDRICH P.R., JAGTAP M., MICHLER C.H., ROMERO-SEVERSON J., 2003 - *Amplification of North American red oak microsatellite markers in European white oaks and Chinese chestnut.* - Silvae Genetica, 52: 176-179.

ALI A.M., ZUBAIR S.J., ABBAS A.M., JUBRAEL J.M., 2016 - *Genetic diversity among Walnuts (*Juglans regia*) population in Kurdistan Region-Iraq using AFLP-PCR.* - ZANCO. Journal of Pure and App. Sci., 28: 50-55.

ALLWRIGHT M.R., TAYLOR G., 2016 - *Molecular breeding for improved second generation bioenergy crops.* - Trends in Plant Sci., 21: 43-54.

AL-MAHMOOD A.A., SHU L., KIM H., RAMIREZ C., PUNG D., GUO Y., LI W., KONG A.N.T., 2016 - *Prostate cancer and chemoprevention by natural dietary phytochemicals.* - Journal of Chin. Pharm. Sci., 25: 633-650.

AL-OKBI S.Y., MOHAMED D.A., HAMED T.E., ESMAIL R.S., DONYA S.M., 2014 - *Prevention of renal dysfunction by nutraceuticals prepared from oil rich plant foods*. - Asian Pac. J. Tropical Biom., 4: 618-626.

ALY M.A., FJELLSTROM R.G., McGRANAHAN G.H., PARFITT D.E., 1992 - *Origin of walnut somatic embryos determined by RFLP and isozyme analysis*. - Hort Science, 27: 61-63.

AMARAL J.S., VALENTÃO P., ANDRADE P.B., MARTINS R.C., SEABRA R.M., 2008 - *Do cultivar, geographical location and crop season influence phenolic profile of walnut leaves?*. - Molecules, 13: 1321-1332.

ANJUM S., GANI A., AHMAD M., SHAH A., MASOODI F.A., SHAH Y., GANI A., 2016 - *Antioxidant and antiproliferative activity of walnut extract (*Juglans regia *L.) processed by different methods and identification of compounds using GC/MS and LC/MS technique*. - J. Food Process and Pres., 41(1): 1-9.

ANSARI A.M., 2015 - *Molecular markers in vegetable improvement*. - Hort. Biotech. Res., 1: 5-10.

APEDA, 2016 - *Walnut.* - Agricultural and Processed Food Products Export Development Authority http://apeda.gov.in/apedawebsite/SubHead_Products/Walnuts.htm.

ARAB L., ANG A., 2015 - *A cross sectional study of the association between walnut consumption and cognitive function among adult us populations represented in*

*NHANES*. - The J. Nutr. Health & Aging, 19: 284-290.

ARULSEKAR S., PARFITT D.E., McGRANAHAN G.H.,1985 - *Isozyme gene markers in* Juglans *species. Inheritance of GPI and AAT in* J. regia *and* J. hindsii. - J. Heredity, 76: 103-106.

ASHRAF M., FOOLAD M.R., 2013 - *Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection*. - Plant Breeding, 132: 10-20.

BABULA P., MIKELOVA R., ADAM V., KIZEK R., HAVEL L., SLADKY Z., 2006 - *Using of liquid chromatography coupled with diode array detector for determination of naphthoquinones in plants and for investigation of influence of pH of cultivation medium on content of plumbagin in* Dionaea muscipula. - J. Chroma B., 842: 28-35.

BAYAZIT S., KAZAN K., GÜLBITTI S., CEVIK V., AYANOĞLU H., ERGÜL A., 2007 - *AFLP analysis of genetic diversity in low chill requiring walnut (*Juglans regia *L.) genotypes from Hatay, Turkey*. - Sci. Hortic., 111: 394-398.

BEHERA U.K., FRANCE J., 2016 - *Chapter four-integrated farming systems and the livelihood security of small and marginal farmers in India and other developing countries*. - Adv. Agr., 138: 235-282.

BILLOT C., RAMU P., BOUCHET S., CHANTEREAU J., DEU M., GARDES L., NOYER J.L., RAMI J.F., RIVALLAN R., LI Y., LU P., WANG T., FOLKERTSMA R.T., ARNAUD E., UPADHYAYA H.D., GLASZMANN J.C., HASH C.T., 2013 - *Massive sorghum collection genotyped with SSR markers to enhance use of global genetic resources* - PLoS ONE, 8(4): 1-16.

BIYIK H., 2010 - *Antimicrobial activity of the ethanol extracts of some plants natural growing in Aydin, Turkey*. - Afr. J. Microbiol. Res., 4: 2318-2323.

BONFILI L., CECARINI V., AMICI M., CUCCIOLONI M., ANGELETTI M., KELLER J.N., ELEUTERI A.M., 2008 - *Natural polyphenols as proteasome modulators and their role as anti-cancer compounds*. - FEBS J., 275: 5512-5526.

BULLÓ M., NOGUÉS M.R., LÓPEZ-URIARTE P., SALAS-SALVADÓ J., ROMEU M., 2010 - *Effect of whole walnuts and walnut-skin extracts on oxidant status in mice*. - Am. J. Cl. Nutr., 26: 823-828.

BYERLEY L.O., SAMUELSON D., BLANCHARD E., LUO M., LORENZEN B.N., BANKS S., TAYLOR C.M., 2017 - *Changes in the gut microbial communities following addition of walnuts to the diet*. - J. Nutr. Bioch., 48: 94-102.

CABEZAS J.A., IBÁÑEZ J., LIJAVETZKY D., VÉLEZ D., BRAVO G., RODRÍGUEZ V., THOMAS M.R., 2011 - *A 48 SNP set for grapevine cultivar identification*. - BMC Plant Biol., 11: 153.

CARVALHO M., FERREIRA P.J., MENDES V.S., SILVA R., PEREIRA J.A., JERÓNIMO C., SILVA B.M., 2010 - *Human cancer cell antiproliferative and antioxidant activities of (*Juglans regia *L.).* - Food Chem. Toxicol., 48: 441-447.

CENAS N., PRAST S., NIVINSKAS H., SARLAUSKAS J., ARNÉR E.S., 2006 - *Interactions of nitroaromatic compounds with the mammalian selenoprotein thioredoxin reductase and the relation to induction of apoptosis in human cancer cells*. - J. Biol. Chem., 281: 5593-5603.

CHAGAS K.P.T., SOUSA R.F., FAJARDO C.G., VIEIRA F.A., 2015 - *Selection of ISSR markers and genetic diversity in a population of* Elaeis guineensis. - Revista Bras. de Ciên. Agr., 10: 147-152.

CHATURVEDI K., SAHIJRAM L., 2015 - *Plant molecular biology applications in horticulture: An overview*, pp. 113-129. - In: BAHADUR B., R. MANCHIKATLA VENKAT, L. SAHIJRAM, and K.V. KRISHNAMURTHY (eds.) *Plant biology and biotechnology. Vol II. Plant genomics and biotechnology*. Springer India, New Delhi, India, pp. 768.

CHEN L., MA Q., CHEN Y., WANG B., PEI D., 2014 - *Identification of major walnut cultivars grown in China based on nut phenotypes and SSR markers*. - Sci. Hort., 168: 240-248.

CHENIANY M., EBRAHIMZADEH H., VAHDATI K., PREECE J.E., MASOUDINEJAD A., MIRMASOUMI M., 2013 - *Content of different groups of phenolic compounds in microshoots of* Juglans regia *cultivars and studies on antioxidant activity.* - Acta Physiol. Plant, 35: 443-450.

CHOI S.W., CHOI J., KIM J., KIM Y., FRISO S., 2016 - *Walnut substantially alters the DNA methylation profile in colon cancer stem cells*. - The FASEB J., 30 (1 Supplement): 912-925.

CHRISTOPOULOS M.V., ROUSKAS D., TSANTILI E., BEBELI P.J., 2010 - *Germplasm diversity and genetic relationships among walnut (*Juglans regia *L.) cultivars and Greek local selections revealed by Inter-Simple Sequence Repeat (ISSR) markers*. - Sci. Hort., 125: 584-592.

CHRISTOPOULOS M.V., TSANTILI E., 2012 - *Storage of fresh walnuts (*Juglans regia *L.) low temperature and phenolic compounds*. - Posthar. Biol. Technol., 73: 80-88.

CHRISTOPOULOS M.V., TSANTILI E., 2015 - *Oil composition in stored walnut cultivars-quality and nutritional value*. - Eur. J. Lipid Sci. Technol., 117: 338-348.

CHRZANOWSKI G., LESZCZYŃSKI B., CZERNIEWICZ P., SYTYKIEWICZ H., MATOK H., KRZYŻANOWSKI R., 2011 - *Phenolic acids of walnut (*Juglans regia *L.)*. - Herba Polonica, 57: 22-29.

CIARMIELLO L.F., PONTECORVO G., PICCIRILLO P., DE LUCA A., CARILLO P., KAFANTARIS I., WOODROW P., 2013 - *Use of nuclear and mitochondrial single nucleotide polymorphisms to characterize English walnut (*Juglans regia *L.) genotypes*. - Plant Mol. Biol. Rep., 31: 1116-1130.

COLARIC M., VEBERIC R., SOLAR A., HUDINA M., STAMPAR F., 2005 - *Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of (*Juglans regia *L.)*. - Agr. Food Chem., 53: 6390-6396.

COSMULESCU S., TRANDAFIR I., NOUR V., 2014 - *Seasonal variation of the main individual phenolics and juglone in walnut (*Juglans regia*) leaves*. - Pharma Biol. J., 52: 575-580.

COSMULESCU S.N., BACIU A., ACHIM G., MIHAI B., TRANDAFIR I., 2009 - *Mineral composition of fruits in different walnut (*Juglans regia *L.) cultivars*. - Not. Bot. Horti. Agrobotanici, Cluj-Napoca, 37: 156.

DANGL G.S., WOESTE K., ARADHYA M.K., KOEHMSTEDT A., SIMON C., POTTER D., McGRANAHAN G., 2005 - *Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut*. - J. Amer. Soc. Hort. Sci., 130: 348-354.

DE LA ROSA R., BELAJ A., MUÑOZ-MÉRIDA A., TRELLES O., ORTÍZ-MARTÍN I., GONZÁLEZ-PLAZA J.J., BEUZÓN C.R., 2013 - *Development of EST-derived SSR markers with long-core repeat in olive and their use for paternity testing*. - J. Amer. Soc. Hort. Sci., 138: 290-296.

DECKELBAUM R.J., TORREJON C., 2012 - *The omega-3 fatty acid nutritional landscape: health benefits and sources*. - The J. Nutr., 142: 587-591.

DESHPANDE R.R., KALE A.A., RUIKAR A.D., PANVALKAR P.S., KULKARNI A.A., DESHPANDE N.R., SALVEKAR J.P., 2011 - *Antimicrobial activity of different extracts of (*Juglans regia *L.) against oral Microflora*. - Int. J. Phar. Pharmaceu. Sci., 3: 200-201.

DO Q.D., ANGKAWIJAYA A.E., TRAN-NGUYEN P.L., HUYNH L.H., SOETAREDJO F.E., ISMADJI S., JU Y.H., 2014 - *Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of* Limno philaaromatica. - J. Food Drug. Anal.., 22: 296-302.

DOĞAN Y., KAFKAS S., SÜTYEMEZ M., AKÇA Y., TÜREMIŞ N., 2014 - Assessment *and characterization of genetic relationships of walnut (*Juglans regia *L.) genotypes by three types of molecular markers*. - Sci. Hort., 168: 81-87.

EBRAHIMI A., FATAHI R., ZAMANI Z., 2011 - *Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia *L.) using morphological traits and SSRs markers*. - Sci. Hort., 130: 146-151.

EBRAHIMI A., ZAREI A., LAWSON S., WOESTE K.E., SMULDERS M.J.M., 2016 - *Genetic diversity and genetic structure of Persian walnut (*Juglans regia*) accessions from 14 European, African, and Asian countries using SSR markers*. - Tree Gen. Genom., 12: 114.

ELSHIRE R.J., GLAUBITZ J.C., SUN Q., POLAND J.A., KAWAMOTO K., BUCKLER E.S., MITCHELL S.E., 2011 - *A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species*. - PLoS ONE, 6(5): 1-10.

ERTURK U., DALKILIC Z., 2011 - *Determination of genetic relationship among some walnut (*Juglans regia *L.) genotypes and their early-bearing progenies using RAPD markers*. - Romanian Biotech. Lett., 16: 5944-5952.

ESKANDARI E., HEIDARIAN E., AMINI S.A., SAFFARI-CHALESHTORI J., 2016 - *Evaluating the effects of ellagic acid on pSTAT3, pAKT, and pERK1/2 signaling pathways in prostate cancer PC3 cells*. - J. Cancer Res. Therap., 12(4): 1266-1271.

FANG W., MEINHARDT L.W., MISCHKE S., BELLATO C.M., MOTILAL L., ZHANG D., 2014 a - *Accurate determination of genetic identity for a single cacao bean, using molecular markers with a nanofluidic system, ensures cocoa authentication*. - J. Agr. Food Chem., 62: 481-487.

FANG W.P., MEINHARDT L.W., TAN H.W., ZHOU L., MISCHKE S., ZHANG D., 2014 b - *Varietal identification of tea (*Camellia sinensis*) using nanofluidic array of single nucleotide polymorphism (SNP) markers*. - Hort. Res., 1: 1-8.

FAROOQUI A., KHAN A., BORGHETTO I., KAZMI S.U., RUBINO S., PAGLIETTI B., 2015 - *Synergistic antimicrobial activity of* Camellia sinensis *and* Juglans regia *against multidrug-resistant bacteria*. - PLoS ONE, 10(2): 1-14.

FARR O.M., TUCCINARDI D., UPADHYAY J., OUSSAADA S.M., MANTZOROS C.S., 2017 - *Walnut consumption increases activation of the insula to highly desirable food cues: A randomized, double-blind, placebo-controlled, cross-over fMRI study*. - Diabetes Obes. Metab., 20(1): 173-177.

FERNÁNDEZ-AGULLÓ A., PEREIRA E., FREIRE M.S., VALENTAO P., ANDRADE P.B., GONZÁLEZ-ÁLVAREZ J., PEREIRA J.A., 2013 - *Influence of solvent on the antioxidant and antimicrobial properties of walnut (*Juglans regia *L.) green husk extracts.* - Indus. Crops Prod., 42: 126-132.

FIGUEROA F., MARHUENDA J., CERDÁ B., ZAFRILLA P., MARTÍNEZ-CACHÁ A., TEJADA L., MULERO J., 2016 - *HPLC-DAD determination and availability of phenolic compounds in 10 genotypes of Walnuts*. - Inter. J. Food Prop., 20: 1-33.

FIRDOUS A.B., SHARMILA G., BALAKRISHNAN S., RAJASINGH P., SUGANYA S., SRINIVASAN N., ARUNAKARAN J., 2014 - *Quercetin, a natural dietary flavonoid, acts as a chemopreventive agent against prostate cancer in an* in vivo *model by inhibiting the EGFR signalling pathway*. - Food Func., 5(10): 2632-2645.

FJELLSTROM R.G., PARFITT D.E., 1995 - *Phylogenetic analysis and evolution of the genus* Juglans *(*Juglandaceae*) as determined from nuclear genome RFLPs.* - Plant System Evol., 197: 19-32.

FJELLSTROM R.G., PARFITT D.E., McGRANAHAN G.H., 1994 - *Genetic relationships and characterization of Persian walnut (*Juglans regia *L.) cultivars using restriction fragment length polymorphisms (RFLPs)*. - J. Amer. Soc. Hort. Sci., 119: 833-839.

FORINO M., STIUSO P., LAMA S., CIMINIELLO P., TENORE G.C., NOVELLINO E., TAGLIALATELA-SCAFATI O., 2016 - *Bioassay-guided identification of the antihyperglycaemic constituents of walnut (*Juglans regia*) leaves*. - J. Func. Foods, 26: 731-738.

FUKUDA T., ITO H., YOSHIDA T., 2004 - *Effect of the walnut polyphenol fraction on oxidative stress in type 2 diabetes mice.* - Biofactors, 21: 251-253.

GANDEV S., 2007 - *Budding and grafting of the walnut (*Juglans regia *L.) and their effectiveness in Bulgaria (review)*. - Bulg. J. Agr. Sci., 13: 683.

GERBER M., 2012 - *Omega-3 fatty acids and cancers: a systematic update review of epidemiological studies*. - B. J. Nutr., 107: 228-239.

GHASEMI K., GHASEMI Y., EHTESHAMNIA A., NABAVI S.M., NABAVI S.F., EBRAHIMZADEH M.A., POURMORAD F., 2011 - *Influence of environmental factors on antioxidant activity, phenol and flavonoids contents of walnut (*Juglans regia *L.) green husks*. - J. Med. Plants Res., 5: 1128-1133.

GHEBRESLASSIE B.M., GITHIRI S.M., TADESSE M., KASILI R.W., 2016 - *Morphological diversity of farmers' and improved potato (*Solanum tuberosum*) cultivars growing in Eritrea*. - J. Plant Studies, 5: 63.

GOVERNMENT OF INDIA, 2015 - *Horticultural Statistics at a Glance*. - Ministry of Agriculture and Farmers Welfare, Department of Agriculture Cooperation and Farmers Welfare, India. http://agricoop.nic.in /imagedefault/hortstat_glance. pdf

GRIEL A.E., KRIS-ETHERTON P.M., HILPERT K.F., ZHAO G., WEST S.G., CORWIN R.L., 2007 - *An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans*. - Nutrition Journal, 6(2): 1-8.

GROSSO G., ESTRUCH R., 2016 - *Nut consumption and age-related disease*. - Maturitas, 84: 11-16.

HAN H., WOESTE K.E., HU Y., DANG M., ZHANG T., GAO X.X., ZHAO P., 2016 - *Genetic diversity and population structure of common walnut (*Juglans regia*) in China based on EST-SSRs and the nuclear gene phenylalanine ammonia-lyase (PAL)*. - Tree Gen. Genom., 12(111): 1-12.

HARDMAN W.E., 2014 - *Walnuts have potential for cancer prevention and treatment in mice*. - The J. Nutr., 144: 555-560.

HAYWARD A.C., TOLLENAERE R., DALTON-MORGAN J., BATLEY J., 2015 - *Molecular marker applications in plants.* - Methods Mol. Biol., 1245: 13-27.

HO H.H., CHANG C.S., HO W.C., LIAO S.Y., LIN W.L., WANG C.J., 2013 - *Gallic acid inhibits gastric cancer cells metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity*. - Toxicol. Appl. Pharmacol., 266: 76-85.

HOSSEINI S., HUSEINI H.F., LARIJANI B., MOHAMMAD K., NAJMIZADEH A., NOURIJELYANI K., JAMSHIDI L., 2014 - *The hypoglycemic effect of* Juglans regia *leaves aqueous extract in diabetic patients: A first human trial*. - DARU, J. Pharma. Sci., 22(1): 1-5.

HSU Y.L., CHANG J.K., TSAI C.H., CHIEN T.T.C., KUO P.L., 2007 - *Myricetin induces human osteoblast differentiation through bone morphogenetic protein-2/p38 mitogen-activated protein kinase pathway*. - Biochem. Pharma., 73: 504-514.

HU Z., ZHANG T., GAO X.X., WANG Y., ZHANG Q., ZHOU H.J., ZHAO P., 2016 - *De novo assembly and characterization of the leaf, bud, and fruit transcriptome from the vulnerable tree* Juglans mandshurica *for the development of 20 new microsatellite markers using Illumina sequencing*. - Mol. Gene. Genom., 291(2): 849-862.

ISHER A.K., KACHROO J., SINGH S.P., BHAT A., 2016 - *Export scenario of dry fruits in Jammu and Kashmir*. - Indian J. Plant Soil, 3: 23-26.

JAKOPIČ J., VEBERIC R., 2009 - *Extraction of phenolic compounds from green walnut fruits in different solvents*. - Acta Agr. Sloven., 93: 11.

JALILI A., SADEGHZADE A., 2012 - *Comparative phenolic profile of Persian walnut (*Juglans regia *L.) leaves cultivars grown in Iran*. - Afr. J. Biochem. Res., 6: 33-38.

JESZKA-SKOWRON M., ZGOŁA-GRZEŚKOWIAK A., STANISZ E., WAŚKIEWICZ A., 2016 - *Potential health benefits and quality of dried fruits: goji fruits, cranberries and raisins*. - Food Chem., 221: 228-236.

JI A., WANG Y., WU G., WU W., YANG H., WANG Q., 2014 - *Genetic diversity and population structure of north China mountain walnut revealed by ISSR*. - Amer. J. Plant Sci., 5: 3194.

JI Y.B., QU Y.Z., ZOU X., CUI L., HU G.J., 2008 - *Growth inhibition and induction of apoptosis in sarcoma 180 cells by Juglone* in vivo, pp. 325-328. - In: QI L. (ed.). *International Seminar on Future BioMedical Information Engineering*. Wuhan, Hubei, China.

JIANG Q., 2014 - *Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy*. - Free Rad. Biol. Med., 72: 76-90.

KAJARABILLE N., DÍAZ-CASTRO J., HIJANO S., LÓPEZ-FRÍAS M., LÓPEZ-ALIAGA I., OCHOA J.J., 2013 - *A new insight to bone turnover: role of-3 polyunsaturated fatty acids*. - The Scien. World J., 2013: 1-16.

KALE A., GAIKWAD S., MUNDHE K., DESHPANDE N., SALVEKAR J., 2010 - *Quantification of phenolics and flavonoids by spectrophotometer from* Juglans regia. - Inter. J. Pharma. Bio. Sci., 1 (3): 1-4.

KALE A.A., GAIKWAD S.A., KAMBLE G.S., DESHPANDE N.R., SALVEKAR J.P., 2011 - In vitro *anthelmintic activity of stem bark of* Juglans regia *L.* - J. Chem. Pharma. Res., 3(2): 298-302.

KARIMI E., JAAFAR H.Z.E., AZIZ M.A., TAHERI S., AZADIGONBAD R., 2014 - *Genetic relationship among* Labisi apumila *(*Myrsinaceae*) species based on ISSR-PCR*. - Gene. Mol. Res., 3: 3301-3309.

KATZ D.L., DAVIDHI A., MA Y., KAVAK Y., BIFULCO L., NJIKE V.Y., 2012 - *Effects of walnuts on endothelial function in overweight adults with visceral obesity: a randomized, controlled, crossover trial*. - J. Amer. Coll. Nutr., 31: 415-423.

KIM H.G., BAE J.H., JASTRZEBSKI Z., CHERKAS A., HEO B.G., GORINSTEIN S., KU Y.G., 2016 - *Binding, antioxidant and anti-proliferative properties of bioactive compounds of sweet paprika (*Capsicum annuum *L.)*. - Plant Foods Human Nutr., 71: 129-136.

KUMAR P., DOLKAR R., MANJUNATHA G., PALLAVI H.M., 2017 - *Molecular fingerprinting and assessment of genetic variations among advanced breeding lines of* Moringa oleifera *L. by using seed protein, RAPD and*

*Cytochrome P 450 based markers*. - South Afr. J. Bot., 111: 60-67.

LEY S.H., HAMDY O., MOHAN V., HU F.B., 2014 - *Prevention and management of type 2 diabetes: dietary components and nutritional strategies.* - The Lancet, 383: 1999-2007.

LI C., LUO S.P., ZENG B., LI J., LI G., 2011 - *Analysis of genetic diversity of germplasm resources of walnut (*Juglans regia *L.) revealed by ISSR in Xinjiang of China.* - Sci. Agr. Sinica, 44: 1871-1879.

LI L., TSAO R., YANG R., LIU C., ZHU H., YOUNG J.C., 2006 - *Polyphenolic profiles and antioxidant activities of heartnut (*Juglans ailanthifolia *var.* cordiformis*) and Persian walnut (*Juglans regia *L.)*. - J. Agr. Food Chem., 54: 8033-8040.

LIAO Z., FENG K., CHEN Y., DAI X., LI S., YIN T., 2014 - *Genome-wide discovery and analysis of single nucleotide polymorphisms and insertions/deletions in* Juglans regia *L. by high-throughput pyrosequencing.* - Plant Omics, 7: 445.

LIU H., YANG W., HOU J., HU N., YIN T, LI S., 2016 a - *Genetic identification of 43 elite clonal accessions of* Populus deltoides *by SSR fingerprinting*. - Canadian J. Plant Sci., 96: 494-502.

LIU W.L., SHIH H.C., WENG I.S., KO Y.Z., TSAI C.C., CHOU C.H., CHIANG Y.C., 2016 b - *Characterization of genomic inheritance of intergeneric hybrids between Ascocenda and Phalaenopsis cultivars by GISH, PCR-RFLP and RFLP*. - PLoS ONE, 11 (4): 1-14.

LONGHI S., GIONGO L., BUTI M., SURBANOVSKI N., VIOLA R., VELASCO R., SARGENT D.J., 2014 - *Molecular genetics and genomics of the* Rosoideae*: state of the art and future perspectives*. - Hort. Res., 1(1): 1-18.

LUCIANO R.L., 2014 - *Acute kidney injury from cherry concentrate in a patient with CKD*. - Amer. J. Kidney Dis., 63: 503-505.

LUGASI A., HÓVÁRI J., SÁGI K.V., BÍRÓ L., 2003 - *The role of antioxidant phytonutrients in the prevention of diseases.* - Acta Biol. Szeged., 47: 119-125.

MA Y., NJIKE V.Y., MILLET J., DUTTA S., DOUGHTY K., TREU J.A., KATZ D.L., 2010 - *Effects of walnut consumption on endothelial function in type 2 diabetic subjects A randomized controlled crossover trial.* - Diab. Care, 33: 227-232.

MAHMOODI R., RAHMANI F., REZAEE R., 2013 - *Genetic diversity among (*Juglans regia *L.) genotypes assessed by morphological traits and microsatellite markers*. - Spanish J. Agr. Res., 11: 431-437.

MALVOLTI M.E., POLLEGIONI P., BERTANI A., MAPELLI S., CANNATA F., 2010 - Juglans regia *provenance research by molecular, morphological and biochemical markers: a case study in Italy*. - Biorem. Biodiv. Bioavail., 4: 84-92.

MALVOLTI M.E., FINESCHI S., PIGLIUCCI M., 1994 - *Morphological integration and genetic variability in* Juglans regia *L.* - J. Hered., 85: 389-394.

MARTÍNEZ M.L., LABUCKAS D.O., LAMARQUE A.L., MAESTRI D.M., 2010 - *Walnut (*Juglans regia *L.) genetic resources, chemistry, by-products*. - J. Sci. Food Agr., 90: 1959-1967.

MARTÍNEZ M.L., MAESTRI D.M., 2008 - *Oil chemical variation in walnut (*Juglans regia *L.) genotypes grown in Argentina*. - Eur. J. Lipid. Sci. Technol., 110: 1183-1189.

MARTÍNEZ M.L., MAESTRI D.M., 2016 - *A perspective on production and quality of argentinian nut oils*, pp. 387-398. - In: KRISTBERGSSON K., and J. OLIVEIRA (eds.) *Traditional foods. Integrating food science and engineering knowledge into the food chain, vol. 10*. Springer, Boston, MA, USA, pp. 405.

McGRANAHAN G., LESLIE C., 1991 - *Walnuts (*Juglans*)*. - Genetic Resources of Temperate Fruit and Nut Crops, 290: 907-974.

McGRANAHAN G.H., LESLIE C., 2009 - *Breeding walnuts (*Juglans regia*)*, pp. 249-273. - In: JAIN S.M., and P.M. PRIYADARSHAN (eds.) *Breeding plantation tree crops: temperate species*. - Springer, New York, USA.

MERTENS-TALCOTT S.U., BOMSER J.A., ROMERO C., TALCOTT S.T., PERCIVAL S.S., 2005 - *Ellagic acid potentiates the effect of quercetin on p21waf1/cip1, p53, and MAP-kinases without affecting intracellular generation of reactive oxygen species* in vitro. - The J. Nutr., 135: 609-614.

MIAH G., RAFII M.Y., ISMAIL M.R., PUTEH A.B., RAHIM H.A., ISLAM K.N., LATIF M.A., 2013 - *A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance*. - Inter. J. Mol. Sci., 14: 22499-22528.

MINISTRY OF AGRICULTURE, 2015 - *Indian Horticulture Database, 2014* - National Horticultural Board, Ministry of Agriculture, Government of India, Gurgaon, India.

MNASRI S.R., SADDOUD O.D., ROUZ S., BEN S., FERCHICHI A., 2017 - *Fingerprinting of the main olive cultivars in Tunisia by morphological and AFLP markers*. - J. New Sci., 37: 2055-2063.

MOUHAJIR F., HUDSON J.B., REJDALI M., TOWERS G.H.N., 2001 - *Multiple antiviral activities of endemic medicinal plants used by Berber peoples of Morocco*. - Pharm. Biol., 39: 364-374.

MUJEEB F., BAJPAI P., PATHAK N., VERMA S.R., 2017 - *Genetic diversity analysis of medicinally important horticultural crop* Aegle marmelos *by ISSR Markers*. - Methods Mol. Biol., 1620: 195-211.

MURADOGLU F., OGUZ H.I., YILDIZ K., 2010 - *Some chemical composition of walnut (*Juglans regia *L.) selections from Eastern Turkey.* - Afr. J. Agr. Res., 5: 2379-2385.

MUTHAIYAH B., ESSA M.M., CHAUHAN V., CHAUHAN A., 2011 - *Protective effects of walnut extract against amyloid beta peptide-induced cell death and oxidative stress in PC12 cells*. - Neurochem. Res., 36: 2096-2103.

NAMGOONG G.M., KHANAL P., CHO H.G., LIM S.C., OH Y.K., KANG B.S., CHOI H.S., 2010 - *The prolyl isomerase Pin1 induces LC-3 expression and mediates tamoxifen resistance in breast cancer*. - J. Biol. Chem., 285: 23829-23841.

NASRI H., BARADARAN A., SHIRZAD H., RAFIEIAN-KOPAEI M., 2015 - *New concepts in neutraceuticals as alternative for pharmaceuticals*. - Inter. J. Prev. Med., 5: 1487-499.

NEGI A.S., LUQMAN S., SRIVASTAVA S., KRISHNA V., GUPTA N., DAROKAR M.P., 2011 - *Antiproliferative and antioxidant activities of* Juglans regia *fruit extracts*. - Pharm. Biol., 49: 669-673.

NICESE F.P., HORMAZA J.I., McGRANAHAN G.H., 1998 - *Molecular characterization and genetic relatedness among walnut (*Juglans regia *L.) genotypes based on RAPD markers*. - Euphytica, 101*:* 199-206.

NJIKE V.Y., AYETTEY R., PETRARO P., TREU J.A., KATZ D.L., 2015 - *Walnut ingestion in adults at risk for diabetes: effects on body composition, diet quality, and cardiac risk measures*. - BMJ Open Diab. Res. Care, 3(1): 1-9.

NOUMI E., SNOUSSI M., HAJLAOUI H., VALENTIN E., BAKHROUF A., 2010 - *Antifungal properties of* Salvadora persica *and* Juglans regia *L. extracts against oral Candida strains*. - Eur. J. Clinc. Microbiol. Infec. Dis., 29: 81-88.

NOUMI E., SNOUSSI M., TRABELSI N., KSOURI R., HAMDANI G., BOUSLAMA L., BAKHROUF A., 2012 - *Antioxidant activities and RP-HPLC identification of polyphenols in the ethyl acetate extract of Tunisian* Juglans regia *L. treated barks*. - J. Med. Plants Res., 6: 1468-1475.

NOUR V., TRANDAFIR I., COSMULESCU S., 2012 - *HPLC determination of phenolic acids, flavonoids and juglone in walnut leaves*. - J. Chrom. Sci. Bms., 51: 883-890.

OLAWUYI O.J., BAMIGBEGBIN B.J., BELLO O.B., 2016 - *Genetic variations on morphological and yields characters of* Celosia argentea *L. germplasm*. - J. Basic Appl. Sci. Int., 13: 160-169.

OLIVEIRA I., SOUSA A., FERREIRA I.C., BENTO A., ESTEVINHO L., PEREIRA J.A., 2008 - *Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia *L.) green husks*. - Food Chem. Toxicol., 46: 2326-2331.

ORHAN I.E., SUNTAR I.P., AKKOL E.K., 2011 - In vitro *neuroprotective effects of the leaf and fruit extracts of* Juglans regia *L. (walnut) through enzymes linked to Alzheimer's disease and antioxidant activity.* - Inter. J. Food Sci. Nutr., 62: 781-786.

PANG K., WOESTE K., MICHLER C., 2017 - *Cultivar identification and genetic relatedness among 25 black walnut (*Juglans nigra*) clones based on microsatellite markers*, pp. 167-182. - In: FEI S., J.L. LHOTKA, J.W. STRINGER, K.W. GOTTSCHALK, and G.W. MILLER (eds.) *Proceedings of the 20th Central Hardwood Forest Conference GTR-NRS-P*. Lexington, KY, USA, Vol. 167.

PAPOUTSI Z., KASSI E., CHINOU I., HALABALAKI M., SKALTSOUNIS L.A., MOUTSATSOU P., 2008 - *Walnut extract (*Juglans regia *L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483*. - British J. Nutr., 99: 715-722.

PARVEEN S., SHAHZAD A., YADAV V., 2016 - *Molecular markers and their application in plant biotechnology*, pp. 389-413*.* - In: SHAHZADA A., S. SHARMA, and S.A. SIDDIQUI (eds.) *Biotechnological strategies for the conservation of medicinal and ornamental climbers*. Springer Inter Publishing, Cham (ZG) Szitzerland, pp. 506.

PAUR I., BALSTAD T.R., KOLBERG M., PEDERSEN M.K., AUSTENAA L.M., JACOBS D.R., BLOMHOFF R., 2010 - *Extract of oregano, coffee, thyme, clove, and walnuts inhibits NF-κB in monocytes and in transgenic reporter mice*. - Cancer. Prev. Res., 3: 653-663.

PEREIRA J.A., OLIVEIRA I., SOUSA A., FERREIRA I.C., BENTO A., ESTEVINHO L., 2008 - *Bioactive properties and chemical composition of six walnut (*Juglans regia *L.) cultivars*. - Food Chem. Toxicol., 46: 2103-2111.

PEREIRA J.A., OLIVEIRA I., SOUSA A., VALENTÃO P., ANDRADE P.B., FERREIRA I.C., ESTEVINHO L., 2007 - *Walnut (*Juglans regia *L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars*. - Food. Chem. Toxicol., 45: 2287-2295.

PERUGU S., VEMULA R., 2014 - *Walnut pedunculagin a probable serm for breast cancer treatment*. - Inter. J. Phar. Pharm. Sci., 7: 233-235.

PITSCHMANN A., ZEHL M., ATANASOV A.G., DIRSCH V.M., HEISS E., GLASL S., 2014 - *Walnut leaf extract inhibits PTP1B and enhances glucose-uptake* in vitro. - J. Ethnopharmacol., 152: 599-602.

POLLEGIONI P., WOESTE K.E., CHIOCCHINI F., OLIMPIERI I., TORTOLANO V., CLARK J., MALVOLTI M.E., 2014 - *Landscape genetics of Persian walnut (*Juglans regia *L.) across its Asian range*. - Tree Gene Genom., 10: 1027-1043.

POP I.F., DORU P., RAICA P., PETRICELE I.V., SISEA C., VAS E., BOTOS B., BODEA M., BOTU M., 2010 - *Assessment of the genetic variability among some* Juglans *cultivars from the Romanian National Collection at SCDP Vâlcea using RAPD markers*. - Rom. Biotechnol. Lett., 15: 41-49.

POP I.F., VICOL A.C., BOTU M., RAICA P.A., VAHDATI K., PAMFIL D., 2013 - *Relationships of walnut cultivars in a germplasm collection: Comparative analysis of phenotypic and molecular data*. - Sci. Horti., 153: 124-135.

POTTER D., GAO F., AIELLO G., LESLIE C., McGRANAHAN G., 2002 - *Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars*. - J. Amer. Soc. Hort. Sci., 127: 75-81.

QAMAR W., SULTANA S., 2011 - *Polyphenols from* Juglans regia *L. (walnut) kernel modulate cigarette extract induced acute inflammation, oxidative stress and lung injury in Wistar rats.* - Hum. Exp. Toxicol., 30: 499-506.

RAFIEIAN-KOPAIE M., NASRI H., 2015 - *On the occasion of world cancer day 2015; the possibility of cancer prevention or treatment with antioxidants: the ongoing cancer prevention researches*. - Inter. J. Preven. Med., 6: 108.

RAHIMIPANAH M., HAMEDI M., MIRZAPOUR M., 2010 -

*Antioxidant activity and phenolic contents of Persian walnut (*Juglans regia *L.) green husk extract*. - Afr. J. Food Sci. Technol., 1: 105-111.

RAHIMZADEH M., JAHANSHAHI S., MOEIN S., MOEIN M.R., 2014 - *Evaluation of alpha-amylase inhibition by* Urti cadioica *and* Juglans regia *extracts*. - Iranian Bas. Med. Sci., 17: 465-469.

RAMPRASATH V.R., AWAD A.B., 2015 - *Role of phytosterols in cancer prevention and treatment*. - J. AOAC Inter., 98: 735-738.

RAO V., 2017 - *Role of SSR markers in characterization of grape (*Vitis vinifera *L.) genotypes and hybrids*. - Inter. J. Agr. Innov. Res., 5: 2319-1473.

REGUEIRO J., SÁNCHEZ-GONZÁLEZ C., VALLVERDÚ-QUERALT A., SIMAL-GÁNDARA J., LAMUELA-RAVENTÓS R., IZQUIERDO-PULIDO M., 2014 - *Comprehensive identification of walnut polyphenols by liquid chromatography coupled to linear ion trap-Orbitrap mass spectrometry*. - Food. Chem., 152: 340-348.

REITER R.J., TAN D.X., MANCHESTER L.C., KORKMAZ A., FUENTES-BROTO L., HARDMAN W.E., QI W., 2013 - *A walnut-enriched diet reduces the growth of LNCaP human prostate cancer xenografts in nude mice*. - Cancer Inves., 31: 365-373.

RIBEIRO A.S., ESTANQUEIRO M., OLIVEIRA M.B., SOUSA LOBO J.M., 2015 - *Main benefits and applicability of plant extracts in skin care products*. - Cosmetics, 2: 48-65.

RINK G., ZHANG G., JINGHUA Z., KUNG F.H., CARROLL E.R., 1994 - *Mating parameters in* Juglans nigra *L. seed orchard similar to natural population estimates*. - Silvae Genet., 43: 261-262.

RUIZ-GARCIA L., LOPEZ-ORTEGA G., DENIA A.F., TOMAS D.F., 2011 - *Identification of a walnut (*Juglans regia *L.) germplasm collection and evaluation of their genetic variability by microsatellite markers*. - Spanish J. Agr. Res., 9: 179-192.

SÁNCHEZ-GONZÁLEZ C., CIUDAD C.J., NOE V., IZQUIERDO-PULIDO M., 2017 - *Health benefits of walnut polyphenols: An exploration beyond their lipid profile*. - Critic. Rev. Food. Sci. Nutr., 57: 3373-3383.

SANEEI P., HASHEMIPOUR M., KELISHADI R., RAJAEI S., ESMAILLZADEH A., 2013 - *Effects of recommendations to follow the dietary approaches to stop hypertension (DASH) diet v. usual dietary advice on childhood metabolic syndrome: a randomised cross-over clinical trial*. - British J. Nutr., 110: 2250-2259.

SCHWINGSHACKL L., HOFFMANN G., MISSBACH B., STELMACH-MARDAS M., BOEING H., 2017 - *An umbrella review of nuts intake and risk of cardiovascular disease*. - Curr. Pharma. Des., 23: 1016-1027.

SHAH T.I., SHARMA E., AHMAD G., 2014 - Juglans regia *Linn: A phytopharmacological review.* - World J. Pharm. Sci., 2 : 364-373.

SHAH T.I., SHARMA E., SHAH G.A., 2015 - *Anti-proliferative, cytotoxicity and anti-oxidant activity of* Juglans regia *extract*. - Amer. J. Cancer Preven., 3: 45-50.

SHAH U.N., MIR J.I., AHMED N., FAZILI K.M., 2016 - *Assessment of germplasm diversity and genetic relationships among walnut (*Juglans regia *L.) genotypes through microsatellite markers*. - J. Saudi Soc. Agr. Sci., 1-12.

SHIMODA H., TANAKA J., KIKUCHI M., FUKUDA T., ITO H., HATANO T., YOSHIDA T., 2008 - *Walnut polyphenols prevent liver damage induced by carbon tetrachloride and d-galactosamine: hepatoprotective hydrolyzable tannins in the kernel pellicles of walnut*. - J. Agr. Food Chem., 56: 4444-4449.

SHIMODA H., TANAKA J., KIKUCHI M., FUKUDA T., ITO H., HATANO T., YOSHIDA T., 2009 - *Effect of polyphenol-rich extract from walnut on diet-induced hypertriglyceridemia in mice via enhancement of fatty acid oxidation in the liver*. - J. Agr. Food Chem., 57: 1786-1792.

SIAHNOURI Z., SADEGHIAN M., SALEHISORMGHI M., QOMI M., 2013 - *Determination of Iranian walnut and pistachio mineral contents*. - J. Basic Appl. Sci. Res., 3: 217-220.

SINGH M., SHUKLA Y., 2015 - *Combinatorial approaches utilizing nutraceuticals in cancer chemoprevention and therapy: a complementary shift with promising acuity*, pp. 185-215. - In: BAGCHI D., SWAROOP A., and M. BAGCHI (eds.) *Genomics, proteomics and metabolomics in nutraceuticals and functional foods*. John Wiley and Sons, Chichester, UK, pp. 686.

SOLAR A., COLARIČ M., USENIK V., STAMPAR F., 2006 - *Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia *L.)*. - Plant. Sci., 170: 453-461.

STAMPAR F., SOLAR A., HUDINA M., VEBERIC R., COLARIC M., 2006 - *Traditional walnut liqueur-cocktail of phenolics.* - Food. Chem., 95: 627-631.

TAPSELL L.C., BATTERHAM M.J., TEUSS G., TAN S.Y., DALTON S., QUICK C.J., CHARLTON K.E., 2009 - *Long-term effects of increased dietary polyunsaturated fat from walnuts on metabolic parameters in type II diabetes*. - European J. Clin. Nutr., 63 :1008-1015.

THAKUR A., 2011 - *Juglone: a therapeutic phytochemical from* Juglans regia *L*. - J. Med. Plants Res., 5: 5324-5330.

TOIVONEN P.M., HODGES D.M., 2011 - *Abiotic stress in harvested fruits and vegetables,* pp. 39-58. - In: SHANKER A., and B. VENKATESWARLU (eds.) *Abiotic stress in plants-mechanisms and adaptations*. InTech China, Shangai, China, pp. 440.

TOPCU H., COBAN N., WOESTE K., SUTYEMEZ M., KAFKAS S., 2015 - *Developing new microsatellite markers in walnut (*Juglans regia *L.) from* Juglans nigra *genomic GA enriched library*. - Ekin J. Crop Breed. Gen., 1-2: 93-99.

TRAVIS R.C., ALLEN N.E., ARMSTRONG M.E.G., BERAL V., CAIRNS B.J., GREEN J., WANG X.S., 2014 - *PP79 Shift work, melatonin and breast cancer risk: review and results from the guernsey and million women study cohorts* - J. Epidem. Comm. Health, 68: A79.

TRIVEDI M.K., BRANTON A., TRIVEDI D., NAYAK G., GANG-

WAR M., JANA S., 2016 - *Molecular analysis of biofield treated eggplant and watermelon crops*. - Adv. Crop Sci. Tech., 4: 208.

TSOUKAS M.A., KO B.J., WITTE T.R., DINCER F., HARDMAN W.E., MANTZOROS C.S., 2015 - *Dietary walnut suppression of colorectal cancer in mice: Mediation by miRNA patterns and fatty acid incorporation*. - The J. Nutr. Biochem., 26: 776-783.

UPADHYAY V., KAMBHOJA S., HARSHALEENA K., DHRUVA K., 2010 - *Anthelmintic activity of the stem bark of* Juglans regia *Linn.* - Res. J. Pharm. Phytochem., 2: 467-470.

USDA, 2016 - *Tree Nuts.* - United States Department of Agriculture, Foreign Agricultural Services, USA.

VAN NOCKER S., GARDINER S.E., 2014 - *Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops.* - Hort. Res., 1(14022): 1-8.

VINSON J.A., CAI Y., 2012 - *Nuts, especially walnuts, have both antioxidant quantity and efficacy and exhibit significant potential health benefits*. - Food Func., 3: 134-140.

VIRTAMO J., TAYLOR P.R., KONTTO J., MÄNNISTÖ S., UTRIAINEN M., WEINSTEIN S.J., ALBANES D., 2014 - *Effects of α-tocopherol and β-carotene supplementation on cancer incidence and mortality: 18-Year postintervention follow-up of the alpha-tocopherol, beta-carotene cancer prevention study.* - Inter. J. Cancer, 135: 178-185.

VYAS D., SHARMA S.K., SHARMA D.R., 2003 - *Genetic structure of walnut genotype using leaf isozymes as variability measure*. - Sci. Hort., 97: 141-152.

WANG H., WU W., PAN G., PEI D., 2015 - *Analysis of genetic diversity and relationships among 86 Persian walnut (*Juglans regia *L.) genotypes in Tibet using morphological traits and SSR markers*. - The J. Hort. Sci. Biotech., 90: 563-570.

WOESTE K., McGRANAHAN G.H., BERNATZKY R., 1996 a - *Randomly amplified polymorphic DNA loci from a walnut backcross [(Juglans* hindsii *×* J. regia*) ×* J. regia*]*. - J. Amer. Soc. Hort. Sci., 121: 358-361.

WOESTE K., McGRANAHAN G.H., BERNATZKY R., 1996 b - *The identification and characterization of a genetic marker linked to hypersensitivity to the cherry leafroll virus in walnut*. - Mol. Breed., 2: 261-266.

WONG A.Y.T., LAI J.M.C., CHAN A.W.K., 2015 - *Regulations and protection for functional food products in the United States.* - J. Funct. Foods, 17: 540-551.

WU B., ZHONG G.Y., YUE J.Q., YANG R.T., LI C., LI Y.J., ZHANG L., 2014 - *Identification of pummelo cultivars by using a panel of 25 selected SNPs and 12 DNA segments*. - PLoS ONE, 9(4): 1-12.

XU H.L., YU X.F., QU S.C., QU X.R., JIANG Y.F., 2012 a - *Juglone, from* Juglans mandshruica *Maxim, inhibits growth and induces apoptosis in human leukemia cell HL-60 through a reactive oxygen species-dependent mechanism.* - Food Chemi. Toxicol., 50: 590-596.

XU J.J., ZHANG L.Y., ZHAO B., SHEN H.F., 2017 - *Assessment of genetic diversity among six populations of* Rhododendron triflorum *in Tibet using ISSR and AFLP markers.* - South Afr. J. Bot., 108: 175-183.

XU Z., HU T., ZHANG F., 2012 b - *Genetic diversity of walnut revealed by AFLP and RAPD markers*. - J. Agr. Sci., 4: 271.

XUE S., ZHANG Z., LIN F., KONG Z., CAO Y., LI C., XU H., 2008 - *A high-density intervarietal map of the wheat genome enriched with markers derived from expressed sequence tags*. - Theor. Appl. Gen., 117: 181-189.

YANG S., GUO N., GE H., 2016 - *Morphological and AFLP-based genetic diversity in* Rosa platyacantha *population in eastern Tianshan mountains of Northwestern China*. - Hort. Plant J., 2: 55-60.

ZHAI M.Z., JING B.N., JIA C.X., LIU C.B., 2007 - *Study on extraction conditions of active antiviral substance from walnut leaves [J]*. - Chem. Indus. Prod., 2: 0-18.

ZHANG W., LIU A., LI Y., ZHAO X., LV S., ZHU W., JIN Y., 2012 - *Anticancer activity and mechanism of juglone on human cervical carcinoma HeLa cells.* - Canadian J. Physiol. Pharmacol., 90: 1553-1558.

ZHAO P., ZHAO G.F., ZHANG S.X., ZHOU H.J., HU Y.H., WOESTE K.E., 2014 - *RAPD derived markers for separating Manchurian walnut (*Juglans mandshurica*) and Japanese walnut (*J. ailantifolia*) from close congeners*. - J. Sys. Evol., 52: 101-111.

ZHU Y., YIN Y., YANG K., LI J., SANG Y., HUANG L., FAN S., 2015 - *Construction of a high-density genetic map using specific length amplified fragment markers and identification of a quantitative trait locus for anthracnose resistance in walnut (*Juglans regia *L.)*. - BMC Gen., 16: 1.

Adv. Hort. Sci., 2018 32(1): 137-142 DOI: 10.13128/ahs-21148

*Short note*

AHS
Advances in Horticultural Science

# Residual effects of bioslurry and amino acids plant biostimulant on carnation (*Dianthus caryophyllus* L.) flower quality

**A.N. Niyokuri [1, 2 (*)], S. Nyalala [2], M. Mwangi [2]**

[1] *Department of Crop Sciences, College of Agriculture, Animal Sciences and Veterinary Medicine (CAVM), University of Rwanda, P.O. Box 210 Musanze, Rwanda.*

[2] *Department of Crops, Horticulture and Soils, Faculty of Agriculture, Egerton University, P.O. Box 536-20115, Egerton, Kenya.*



*Key words*: bioslurry, carnation, flower quality, plant biostimulant, residual effect.

**(*) Corresponding author:**
nnari26@gmail.com

**Citation:**
NIYOKURI A.N., NYALALA S., MWANGI M., 2018 - *Residual effects of bioslurry and amino acids plant biostimulant on carnation (*Dianthus caryophyllus *L.) flower quality.* - Adv. Hort. Sci., 32(1): 137-142



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 7 August 2017
Accepted for publication 23 October 2017

**Abstract: A greenhouse experiment was conducted in Finlays, Lemotit Flower Farm in Kenya to determine the residual effect of bioslurry and an amino acids plant biostimulant on carnation flower quality. A second flush of carnation plants growing in previous experimental plots was used. This experiment was laid out in a split plot design with three replications. Four levels of bioslurry: 0, 0.125, 0.25 and 0.5 L $m^{-2}$ were applied in the main plots while four levels of plant biostimulant: 0, 2.0, 2.5 and 3.0 L $ha^{-1}$ were used in the sub-plots. Results showed that there was no significant residual effect of bioslurry on studied parameters. Residual effects of plant biostimulant applied at 2.0, 2.5 and 3.0 L $ha^{-1}$ resulted in a significant increase in carnation flower stalk length by 1.08 to 1.72 cm compared to control. However, there was negligible reduction of the flower stalk diameter (0.1 mm) and no significant residual effect of plant biostimulant on flower head size. Moreover, there were no residual interactive effects of bioslurry and plant biostimulant on studied parameters. These results suggest that plant biostimulant can be used to improve the flower stem length in the subsequent flush of carnation plants supplied with a full dose of inorganic fertilizers.**

## 1. Introduction

Carnation *(Dianthus caryophyllus* L.) is a popular cut-flower throughout the world (Roychowdhury and Tah, 2011). It is preferred to other cut flowers in several exporting countries as it lasts longer after being cut, has a wide range of attractive forms and colours, has the ability to withstand long distance transportation and significant ability to rehydrate after continuous shipping (Salunkhe *et al.,* 1990; Kanwar and Kumar, 2009;

Renukaradya *et al.,* 2011).

Although it is indigenous to the Mediterranean region, carnation can be grown in almost every climate in glasshouses, plastic houses, and shade nets as well as in open field (Aydinsakir *et al.,* 2011). It was fourth among the top ten imported cut flower to the Netherlands after roses, St John's wort (*Hypericum* spp.) and gypsophila with a turnover of €18 million (FloraHolland, 2017). It was also one of the main cut flowers exported by Kenya in 2012 (CBI, 2013) and among the leading cut flowers locally used in flower arrangements and in value addition of flowers, in form of bouquets. In 2014, carnations contributed 4% of the domestic value of floriculture in Kenya (HCD, 2015).

The quality of carnations is currently affected by many problems such as calyx splitting and short stem length for some varieties. To meet the required quality parameters such as stem length and girth, flowers size and number, farmers resort to heavy application of inorganic fertilizers and synthetic plant growth regulators. Although this results in increased production and quality, it adversely affects soil productivity and the environment. This is because pesticides, phosphorus and nitrate used in carnation production represent the major agricultural pollutants that threaten the environment (Nardi *et al.,* 2016).

With the increasing relevance of social and environmental standards, current research is focusing on developing alternative systems in crop production through development of unconventional and non-pollutant solutions. Currently, there are limited organic alternatives to meet plant nutrients. Moreover, organic alternatives for plant growth regulators are lacking in many crops and limited in other crops such as carnations.

Use of compost derived from plants and/or animal wastes as soil amendment or fertilizer additive has been reported as an alternative in the production of several ornamental plants. Moreover, bioslurry, the residual manure generated through anaerobic decomposition of various organic materials is considered a quality organic fertilizer (Islam, 2006). Similarly, the use of biostimulants in sustainable agriculture has been growing particularly for their capacity of enhancing nutrition efficiency and stress response (du Jardin, 2012). Biostimulants can be obtained from different organic materials and include complex organic materials, humic substances, beneficial chemical elements, peptides and amino acids (protein hydrolysates), seaweed extracts, inorganic salts, chitin and chitosan derivatives, antitranspirants, amino acids and other N-containing substances (du Jardin, 2015; Nardi *et al.,* 2016).

Many studies (Karki, 2001; Islam, 2006; Shahbaz, 2011; Jeptoo *et al*., 2013; Shahariar *et al.,* 2013) reported yield increases and quality improvement on many crops such as Okra (*Hibiscus esculentus* L.), maize (*Zea mays*), cabbage (*Brassica oleracea* var. *capitata)* and carrot (*Daucus carota*) following bioslurry application. Studies by Nahed *et al.* (2009 a, b), Paradiković *et al*. (2011) and Mondal *et al.* (2015) reported that plant biostimulants could be successfully used in the production of ornamental and other horticultural crops such as *Antirrhinum majus*, Gladiolus (*Gladiolus grandflorum* L.) sweet yellow pepper (*Capsicum annuum* L.) and *Eustoma grandiflorum*.

However, many studies only evaluated the bioslurry and biostimulant direct effects, whereas there are only a few studies which focused on their residual effects on the quality and yield of many crops including carnations. This study was therefore aiming at evaluating residual effects of bioslurry and plant biostimulant on flower quality in carnation plants which previously received applications of bioslurry and plant biostimulant.

## 2. Materials and Methods

*Experiment location*

The study was conducted in a greenhouse at Lemotit flower farm of Finlays Horticulture Kenya Ltd. situated in Kenya at latitude 0° 22' South and longitude 35° 18' East, from March to September 2015.

*Experimental design and treatments application*

Carnation 'Walker' plants planted on soil media at a density of 36 plants per $m^2$ were used. These plants had received drench applications of bioslurry and plant biostimulant, four times at bi-weekly intervals after pinching (three weeks after transplanting) during the period September 2014 to February 2015. The experimental design was a split-plot design with three replications. The main plot measured 5.5 x 1 m (5.5 $m^2$) while the sub-plot was 1 x 1 m (1 $m^2$). Buffer zone of 0.5 m and 1 m separated inter-plots and individual main blocks respectively. Cow dung bioslurry was applied in main plots at the rate of 0.125, 0.25, 0.5 L $m^{-2}$ and control diluted in one litre of water prior to application. Rates of plant biostimulant used were 2.0, 2.5 and 3.0 L $ha^{-1}$ and control thoroughly mixed with water at the rate of 5000 L $ha^{-1}$ and they

were applied to the sub-plots during the period of September 2014 to February 2015.

Bioslurry used had at wet basis a pH of 7.44, 0.23% of N, 4.58 ppm of P, 89.3 ppm of K, 4.31 ppm of Ca, 19.91 ppm of Mg and density of 1.0195 kg $L^{-1}$. The plant biostimulant used was Hicure®, an amino acids based plant biostimulant. This plant biostimulant contains a balanced mixture of free amino acids (with higher proline and glycine contents) and peptides (hydrolysed protein) of natural origin. It is composed of amino acids and peptides (62.5%), total nitrogen (10.9%) and organic carbon (29.4%). After harvest in February 2015, carnation plants were allowed to grow for subsequent season in order to study the residual effects of bioslurry and plant biostimulant.

*Maintenance practices*

All treatments benefited from a weekly application of mineral fertilizers through fertigation using: 3.06 g N, 3.51 g $P_20_5$, 5.19 g $K_20$, 1.71 g Ca and 0.74 g Mg, plus trace elements per square metre. Routine crop management practices included irrigation, supporting, weeding, training, disbudding and pest management. Harvesting was done at the paint brush stage when petals started to elongate outside the calyx.

*Data collection and analysis*

Data were collected from 10 tagged sample plants per sub-plot on three flower quality parameters namely the length of flower stalk, diameter of flower stalk and flower head size (diameter and head length). The length of flower stalk was measured in centimetres from the point just below the bud to the point of origin of branch on the main stem at harvest; diameter of flower stem was measured in millimetres using digital vernier callipers. The flower head length was recorded in millimetres from the point just below the calyx to the upper point of the flower while the flower head diameter was recorded in millimetres at harvesting from each harvested cut flower at paint brush stage using digital vernier calliper. All data were subjected to analysis of variance (ANOVA) using GENSTAT 14th Edition. Separation of means was performed using the Tukey's Honest Significant Difference (HSD) test at $P \leq 0.05$.

## 3. Results

There was no significant residual effect of bioslurry on measured parameters (Table 1). However, a significant residual effect of plant biostimulant on plants which had received any level of plant biostimulant was observed on flower stalk length and flower stalk diameter (Table 2). Carnations plants that had received 2.0, 2.5 and 3.0 L of plant biostimulant had significantly longer flower stalks and significantly thinner flower stalks compared to the control (Table 2).

The application of different rates of plant biostimulant did not show a significant residual effect on flower head size (diameter and head height) as presented in table 2. The interaction between different levels of bioslurry and those levels of plant biostimulant had no significant residual effects on measured parameters (Table 3).

Table 1 - Residual effect of bioslurry on carnation flower quality

| Bioslurry levels (L $m^{-2}$) | Flower stalk length (cm) | Flower stalk diameter (mm) | Flower head diameter (mm) | Flower head length (mm) |
|---|---|---|---|---|
| 0 | 81.01 | 5.52 | 21.77 | 40.58 |
| 0.125 | 81.18 | 5.56 | 22.04 | 40.44 |
| 0.25 | 81.02 | 5.55 | 21.86 | 40.56 |
| 0.5 | 81.15 | 5.55 | 21.81 | 40.48 |

Table 2 - Residual effect of plant biostimulant on carnation flower quality

| Levels of plant biostimulant (L $ha^{-1}$) | Flower stalk length (cm) | Flower stalk diameter (mm) | Flower head diameter (mm) | Flower head length (mm) |
|---|---|---|---|---|
| 0 | 80.12 b* | 5.628 a | 21.97 | 40.53 |
| 2 | 81.20 a | 5.512 b | 21.83 | 40.47 |
| 2.5 | 81.20 a | 5.515 b | 21.74 | 40.58 |
| 3 | 81.84 a | 5.528 b | 21.93 | 40.48 |

* Means in the same column with the same letter are not significantly different at $P \leq 0.05$ using Tukey's HSD test.

## 4. Discussion and Conclusions

Results of this study showed a significant increase of flower stalk length as a result of the residual effect of plant biostimulant. This increase of flower stalk length may be as a result of enhancement of macro nutrient uptake by plant biostimulant (Calvo *et al.,* 2014; Rose *et al.,* 2014) which rapidly improves the growth compared to treatments without plant biostimulant. The other probable reason would be the direct uptake of amino acids which are immediately used by carnation plants for their growth and devel-

Table 3 - Residual effects of the interaction of bioslurry and plant biostimulant on carnation flower quality

| Bioslurry levels (L $m^{-2}$) | Level of plant bio-stimulant (L $ha^{-1}$) | Flower stalk length (cm) | Flower stalk diameter (mm) | Flower head diameter (mm) | Flower head length (mm) |
|---|---|---|---|---|---|
| 0 | 0 | 79.94 | 5.57 | 21.81 | 40.61 |
| | 2 | 81.07 | 5.48 | 21.74 | 40.57 |
| | 2.5 | 80.83 | 5.51 | 21.71 | 40.45 |
| | 3 | 82.21 | 5.51 | 21.8 | 40.70 |
| 0.125 | 0 | 79.75 | 5.65 | 22.13 | 40.55 |
| | 2 | 81.30 | 5.53 | 21.81 | 40.46 |
| | 2.5 | 81.68 | 5.51 | 21.79 | 40.67 |
| | 3 | 81.97 | 5.56 | 22.44 | 40.08 |
| 0.25 | 0 | 80.28 | 5.67 | 22.06 | 40.49 |
| | 2 | 81.14 | 5.52 | 21.95 | 40.50 |
| | 2.5 | 80.85 | 5.50 | 21.76 | 40.60 |
| | 3 | 81.82 | 5.51 | 21.69 | 40.64 |
| 0.5 L | 0 | 80.52 | 5.61 | 21.88 | 40.47 |
| | 2 | 81.29 | 5.52 | 21.84 | 40.35 |
| | 2.5 | 81.44 | 5.55 | 21.7 | 40.58 |
| | 3 | 81.35 | 5.53 | 21.81 | 40.41 |

opment (Calvo *et al.,* 2014). There are strong evidences that the increase in flower stalk length may be attributed to residual auxins and gibberellins like activities of the plant biostimulant (Brown and Saa, 2015). In fact, both auxins and gibberellins are important in plant cell division and elongation (Ertani *et al.*, 2009; Calvo *et al.,* 2014 and Colla *et al.,* 2014). Results of this study are in agreement with previous findings by Ertani *et al.* (2009) and Colla *et al.* (2014). Colla *et al.* (2014) reported an increase in coleoptile elongation rate when compared to the control, in a dose-dependent fashion, comparable with the effects of indole-3-acetic acid following the treatment of maize with the protein hydrolysate. The same study provided additional evidences of a gibberellin-like activity of protein hydrolysate when application of plant-derived protein hydrolysate "Trainer" at all doses significantly increased the shoot length of the gibberellins deficient dwarf pea plants by an average value of 33% in comparison with the control treatment. Similar results previously reported by Ertani *et al.* (2009) showed that the treatment of lettuce with both protein hydrolysate based fertilizers resulted in an increase in the epicotyl length comparable with the effects of exogenous gibberellic acid. The occurrence of this residual effect was perhaps the result of previous down-regulation mechanisms which affected the effect of plant biostimulant in the previous season. Ammonium, which was used as a source of nitrogen, has been reported to down-regulate amino acids (Henry and Jefferies, 2003 cited by Gioseffi *et al.,* 2012; Thornton and Robinson, 2005 cited by Gioseffi *et al.,* 2012).

The observed reduction in flower stalk diameter is suspected to be a gibberellin-like activity which promoted the flower stalk length (Ertani *et al.*, 2009; Colla *et al.*, 2014) at the expense of flower stalk diameter.

The absence of residual effect of plant biostimulant on flower head size was possibly due to constant supply of nutrients through fertigation. The flower head size is usually a result of carbohydrates stored for subsequent growth and reproductive processes (Islam *et al.,* 2010). Although there are evidences that biostimulants can enhance macro nutrient uptake (Calvo *et al.,* 2014; Rose *et al.,* 2014), it is possible that the residual effect of plant biostimulant was limited to stimulating the elongation of flower stalk. Hence, the residual effect may have contributed much during early stages when carnation plants started re-growing after the harvest by improving growth and nutrients assimilation as previously revealed by Colla *et al.* (2014).

Bioslurry did not show any significant residual effect on the flower stalk length, flower stalk diameter and the flower head size. Generally, nutrients in cow dung slurry and poultry manure slurry are released in higher amounts compared to their original state (Haque *et al.,* 2015). This is because bioslurry, with its narrower C:N than farmyard manure, shows better results on soil nutrient availability at early stages of its application while, farm yard manure affects the nutrient uptake to the plant in more consistent manner because its mineralization occurs at later stages (Muhmood *et al*., 2014). However, results of our study are not in agreement with Shahzad *et al.* (2015) who reported that the application of bioslurry and composted poultry manure as a bio-fertilizer improves soil organic matter contents and availability of soil nutrients (N, P and K) to the subsequent crop, resulting in increased crop productivity and reducing the cost of fertilizer to subsequent crop. In fact, the residual effect of bioslurry may depend on its initial content, characteristics and the quantity applied and for these reasons, the residual effect of bioslurry on carnation grown in the following season was very limited.

Based on results of this study, we can conclude that the application of plant biostimulant on carnations plants supplied with a full dose of inorganic fer-

tilizers may have residual effect on the subsequent production flush. However, the residual effect of bioslurry on carnations grown in the same conditions may depend on its nutrient content and physico-chemical characteristics. Further works would be necessary to study the application of both products under lower rates of inorganic fertilizers and extend over many productions flushes to find out the extent of their residual effects.

**Acknowledgements**

The authors are thankful to James Finlays Company for providing the research plant materials and allowing us to conduct experiments in their Lemotit farm.

**References**

AYDINSAKIR K., TUZEL I.H., BUYUKTAS D., 2011 - *The effects of different irrigation levels on flowering and flower quality of carnation (*Dianthus caryophyllus *L.) irrigated by drip irrigation*. - Afr. J. Biotechnol., 10(66): 14826-14835.

BROWN P., SAA S., 2015 - *Biostimulants in agriculture*. - Front. Plant Sci., 6: 671.

CALVO P., NELSON L., KLOEPPER J.W., 2014 - *Agricultural uses of plant biostimulants*. - Plant Soil, 383: 3-41.

CBI, 2013 - *CBI Tradewatch cut flowers and foliage*. *Survey report*. - CBI, Ministry of Foreign Affairs, www.cbi.eu.

COLLA G., ROUPHAEL Y., CANAGUIER R., SVECOVA E., CARDARELLI M., 2014 - *Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis*. - Front. Plant Sci., 5: 448.

DU JARDIN P., 2012 - *The science of plant biostimulants. A bibliographical analysis*. - Final Report "Ad Hoc study report on bio-stimulants products", pp. 1-37.

DU JARDIN P., 2015 - *Plant biostimulants: Definition, concept, main categories and regulation*. - Sci. Hort., 196: 3-14.

ERTANI A., CAVANI L., PIZZEGHELLO D., BRANDELLERO E., ALTISSIMO A., CIAVATTA C., NARDI S., 2009 - *Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings*. - J. Plant Nutr. Soil Sci., 172: 237-244.

FLORAHOLLAND, 2017 - *Facts and figures 2016*. - https://www.royalfloraholland.com/en.

GIOSEFFI E., DE NEERGAARD A., SCHJOERRING J.K., 2012 - *Interactions between uptake of amino acids and inorganic nitrogen in wheat plants*. - Biogeosciences, 9: 1509-1518.

HAQUE M.A, JAHIRUDDIN M., RAHMAN M.M., SALEQUE M.A., 2015 - *Phosphorus mineralization of bioslurry and other manures in soil.* - J. Environ. Waste Manag., 2(2): 79-83.

HCD, 2015 - *Horticulture. Validated report 2014*. - Horticulture Crops Directorate, pp. 1-68.

ISLAM M.R., RAHMAN S.M.E., RAHMAN M., OH D.H., RA C.S., 2010 - *The effects of biogas slurry on the production and quality of maize fodder.* - Turk. J. Agric. For., 34: 91-99.

ISLAM M.S., 2006 - *Use of bioslurry as organic fertilizer in Bangladesh agriculture*. - International Workshop on the use of bioslurry, Domestic biogas programmes, 27-28 September, Bangkok, Thailand.

JEPTOO A., AGUYOH J.N., SAIDI M., 2013 - *Improving carrot yield and quality through the use of bio-slurry manure.* - Sustainable Agric. Res., 2(1): 164-172.

KANWAR J.K., KUMAR S., 2009 - *Influence of growth regulators and explants on shoot regeneration in carnation*. - Hort. Sci. (Prague), 36(4): 140-146.

KARKI K.B., 2001 - *Response to bio-slurry application on maize and cabbage in Laliptur District*. - Final Field Research Report Submitted to Alternative Energy Promotion Centre of Ministry of Science and Technology, Pulchok, Nepal.

MONDAL M.F., ASADUZZAMAN M., TANAKA H., ASAO T., 2015 - *Effects of amino acids on the growth and flowering of* Eustoma grandiflorum *under autotoxicity in closed hydroponic culture.* - Sci. Hort., 192: 453-459.

MUHMOOD A., JAVID S., AHMAD Z.A., MAJEED A., RAFIQUE R.A., 2014 - *Integrated use of bioslurry and chemical fertilizers for vegetable production.* - Pakistan J. Agric. Sci., 51(3): 565-570.

NAHED G.A.A., LOBNA S.T., SAAD M.M. I., 2009 a - *Some studies on the effect of putrescine, ascorbic acid and thiamine on growth, flowering and some chemical constituents of Gladiolus plants at Nubaria.* - Ozean J. Appl. Sci., 2(2): 169-179.

NAHED G.A.A., MONA H.M., AZZA A.M.M., 2009 b - *Physiological effect of phenylalanine and tryptophan on the growth and chemical constituents of* Antirrhinum majus *plants*. - Ozean J. Appl. Sci., 2(4): 399-407.

NARDI S., PIZZEGHELLO D., SCHIAVON M., ERTANI A., 2016 - *Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism*. - Sci. Agr., 73(1): 18-23.

PARADIKOVIĆ N., VINKOVIĆ T., VRČEK I.V., ŽUNTAR I., BOJIĆ M., MEDIĆ-ŠARIĆ M., 2011 - *Effect of natural biostimulants on yield and nutritional quality: an example of sweet yellow pepper (*Capsicum annuum L.*) plants*. - J. Sci. Food Agr., 91: 2146-2152.

RENUKARADYA S., PRADEEPKUMAR C.M., SANTHOSHA H.M., DRONACHARI M., SHASHIKUMAR R.S., 2011 - *Effect of integrated system of plant nutrition management on growth, yield and flower quality of carnation* (Dianthus caryophyllus *L.*) *under green house*. - Asian J. Hort., 6(1): 106-112.

ROSE M.T., PATTI A.F., LITTLE K.R., BROWN A.L., JACKSON W. R., CAVAGNARO T.R., 2014 - *A meta-analysis and*

*review of plant-growth response to humic substances: practical implications for agriculture*. - Adv. Agron., 124: 37-89.

ROYCHOWDHURY R., TAH J., 2011 - *Evaluation of genetic parameters for agro-metrical characters in carnation genotypes*. - Afr. Crop Sci. J., 19(3): 183-188.

SALUNKHE D.K., BHAT N.R., DESAI B.B., 1990 - *Postharvest biotechnology of flowers and ornamental plants*. - Springer Verlag, New York, USA, pp. 28-46.

SHAHABZ M., 2011 - *Potential of bio-slurry and compost at different levels of inorganic nitrogen to improve growth and yield of okra (*Hibiscus esculentus *L.).* - M.S. Thesis, University of Agriculture Faisalabad, Pakistan.

SHAHARIAR M.S., MONIRUZZAMAN M., SAHA B., CHAKRABORTY G., ISLAM M., TAHSIN S., 2013 - *Effects of fresh and digested cowdung and poultry litter on the growth and yield of cabbage (*Brassica oleracea*).* - Bangladesh J. Sci. Ind. Res., 48(1): 1-6.

SHAHZAD K., KHAN A., SMITH J.U., SAEED M., KHAN S.A., KHAN S.M., 2015 - *Residual effects of different tillage systems, bioslurry and poultry manure on soil properties and subsequent wheat productivity under humid subtropical conditions of Pakistan*. - Intl. J. Biosci., 6 (11): 99-108.

Adv. Hort. Sci., 2018 32(1): 143-147 DOI: 10.13128/ahs-22715

*Short note*

AHS
Advances in Horticultural Science

# Comparative analysis of volatile compounds (potential aromatic ability) in the fruit of 15 olive Italian cultivars

**C. Taiti [1], M. Redwan [1], E. Marone [2 (*)], G. Atzori [1], E. Azzarello [1], S. Mancuso [1]**

[1] *Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università degli studi di Firenze, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy.*

[2] *Facoltà di Bioscienze e Tecnologie Agro-Alimentari e Ambientali, Università degli Studi di Teramo, Via R. Balzarini, 1, 64100 Teramo, Italy.*



*Key words:* olive fruits, PTR-ToF-MS, volatile organic compounds (VOCs).

**Abstract: Virgin olive oils (VOOs) are characterized by peculiar flavors appreciated by the consumers all over the world. Their organoleptic characteristics depend on the aromatic properties of the fruits of the different cultivars, which will originate the final products. VOCs spectra of fifteen certified Italian olive cultivars of the University of Florence Germplasm collection, chosen as their different geographical origin, diffusion, and product purpose, were acquired using a Proton Transfer Reaction Time-of-Flight Mass Spectrometer (PTR-ToF-MS). The VOCs analyses highlighted a great variability among the fifteen cultivars, mostly due to compounds (C6 and C5) deriving from polyunsaturated fatty acids through the LOX pathway. The early identification in the olive fruit of these compounds which are considered among the major contributors to the positive VOOs attributes, would be useful to produce high quality olive oils, and get useful information to individuate the best parents for the genetic improvement.**

**(*) Corresponding author:**
emarone@unite.it

**Citation:**
TAITI C., REDWAN M., MARONE E., ATZORI G., AZZARELLO E., MANCUSO S., 2018 - *Comparative analysis of volatile compounds (potential aromatic ability) in the fruit of 15 olive Italian cultivars.* - Adv. Hort. Sci., 32(1): 143-147



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 20 February 2018
Accepted for publication 6 March 2018

## 1. Introduction

Virgin olive oil is worldwide considered a "commodity", and until the Second World War, a strategic food (Fiorino *et al*., 2010). The complex flavor of virgin olive oil is mainly produced by volatile organic compounds (VOCs) whose formation is related to olive fruit cell destruction (Morales *et al*., 1996; Angerosa, 2004). The total amount and types of VOCs emitted change during the processing steps of olive fruit in the olive mill (Morales *et al*., 1997). In particular, VOCs emission is linked to the destruction of the cell structure of olive fruits which activates a specific chain of enzymatic reaction (LOX cascade) (Angerosa *et al*., 2004). The C6 and C5 are the compounds which most affect the aroma of olive oil gen-

erating the positive attributes such as fruity and herbaceous notes (Marone *et al.*, 2017). These compounds are usually responsible for typical aromas and flavors and play a dominant role in determining the peculiar aroma and quality of olive oils (Zunin *et al.*, 2005). The synthesis of C5 and C6 compounds is linked to the level of LipOXygenase (LOX) activity to be catabolized through its pathway during the mill process (Garçia-Vico *et al.*, 2017). Different C6 straight chains are produced by the action of hydroperoxide lyase on polyunsaturated C18 fatty acids (linoleic C18:2 and linolenic C18:3). While, C5 seem to be synthesized through another branch of the LOX pathway starting from 13-hydroperoxides derived from linolenic acid (Garçia-Vico *et al*., 2017).

Currently, the most innovative analytical technique used to detect VOCs emitted by fruits, which provides a high resolution coupled to a rapid screening power of samples and easy to handle without sample manipulation, is the Proton Transfer Reaction-Time of Flight-Mass Spectrometer (PTR-ToF-MS) (Mayr *et al*., 2002; Masi *et al*., 2015; Taiti *et al*., 2017 a).

Currently, a little information is available and at the best of our knowledge one study was carried out to understand the VOCs profile emission from olive fruits related to two cultivars (Masi *et al.*, 2015). The aim of this study was to develop a quick, accurate and relatively simple evaluation method based on the PTR-MS technique, to define the aromatic potentiality of each olive cultivar starting from olive fruits.

This method could potentially help to address the choice of the cultivars in new plantations and to identify parents for future crosses, aimed to improve some organoleptic characteristics of the oils.

## 2. Materials and Methods

*Plant material*

Fruits of 15 certified Italian olive cultivars, clonally propagated, different in their geographical origin, distribution and fruit's use were obtained from plants of Olive Germplasm collection deriving from the Italian and World olive Gene bank (Montepaldi experimental farm (43° 40' 39'' North latitude, 11° 08' 46" East longitude, 210 m asl), University of Florence) (Table 1). The rainfed, bush trained, fourteen years old olive plants are ordinarily cultivated, grown on a slight slope of a sedimentarious gipsy-arenaceous soil; the annual rainfall average is 867 mm, the average annual temperature results 13.3°C.

*Fruit sampling*

For each cultivar, 1 kg of sound and healthy fruits was collected on October 20$^{th}$ from different parts of the trees of each cultivar. The day after, 100 washed fruits from each cultivar were weighted (W) and the color index (CI) was determined (Uceda and Hermoso, 1998). Subsequently, for each cultivar nine whole and cut samples of fruits (~10 g) were submitted to the VOCs analysis.

*Statistical analyses*

One way analysis of variance (ANOVA) was per-

Table 1 - List of the characteristics of the genetic material

| Cultivar | Origin | Product use | Fruit average weight (g) | Color Index (0-7) |
|---|---|---|---|---|
| Ascolana tenera | Marche | Table | 4.80 ± 0.51 | 2.2 |
| Bianchera | Friuli Venezia Giulia | Olive oil | 2.68 ± 0.12 | 1.5 |
| Carolea | Calabria | Olive oil/Table | 3.91 ± 1.17 | 1.2 |
| Coratina | Apulia | Olive oil | 2.05 ± 0.60 | 1.5 |
| Fasolona | Basilicata | Olive oil/Table | 3.80 ± 0.58 | 3.1 |
| Frantoio | Tuscany | Olive oil | 1.96 ± 0.36 | 1.5 |
| Itrana | Lazio | Olive oil/Table | 2.19 ± 0.94 | 1.4 |
| Leccino | Tuscany | Olive oil | 2.02 ± 0.19 | 2.2 |
| Maiatica di Ferrandina | Basilicata | Olive oil/Table | 2.75 ± 0.93 | 1.2 |
| Moraiolo | Tuscany | Olive oil | 1.42 ± 0.28 | 2.1 |
| Nocellara del Belice | Sicily | Olive oil/Table | 3.84 ± 0.76 | 1.8 |
| Palmarola | Apulia | Olive oil/Table | 2.15 ± 0.72 | 3.1 |
| Sant'Agostino | Apulia | Table | 4.36 ± 0.81 | 0.7 |
| Santa Caterina | Tuscany | Table | 5.80 ± 0.48 | 0.4 |
| San Francesco | Tuscany | Table | 2.68 ± 0.22 | 1.5 |

formed to compare the considered groups of chemical compounds: C5, C6, other VOCs, and total VOCs. Separation of means was performed by the Fisher's LSD test (p = 0.01). Computations were performed by Statgraphics Centurion XV v. 15.0.04.

A Factor Analysis (FA) was applied to the spectral data of 273 olive oil samples, considering as factors three grouping of compounds: total VOCs, C5, and C6, respectively. Computations were performed by XLSTAT 2014.5.03.

A Principal Component Analysis (PCA, unsupervised method) was applied to the whole spectral data of 273 olive oil samples, submitted to a logarithmic transformation and mean centering as pre-processing. Computations were performed by PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB_ R2015b (Mathworks Inc., Natick, MA, USA).

*Volatile compounds detection*

Through a PTR-ToF-8000 (IONICON Analytik, GmbH, Innsbruck, Austria) measurements were performed in a similar way to the one reported by Taiti *et al.* (2017 b). Each analyzed sample consisted of ~10 g of olive fruits intact or cut into 4 parts. Subsequently the sample was inserted into a 3/4 L glass container plugged with a cover in which two Teflon tubes are inserted, connected respectively to a zero-air generator (Peak Scientific) and to the PTR-ToF-MS. The drift tube had the following ionization conditions: pressure of 2.20 mbar, voltage 600V and temperature 110°C. Mass spectrometric data were collected over a mass range of *m/z* 21 to 210 and the acquisition time for each samples was 0.1 ns, for 120 seconds. The instrument internal calibration was based on: *m/z* = 29.997 ($NO^+$); *m/z* = 59.049 ($C_3H_7O^+$) and *m/z* = 137.132 ($C_{10}H_{17}^+$) and was performed off-line. The raw data were acquired with the TofDaq Software (Tofwerk AG, Switzerland) as cps (count per second), and subsequently were converted in ppbv following the formula described by Lindinger and Jordan (1998) on the basis of the primary ion signal.

## 3. Results and Discussion

The whole intact olive fruits of the fifteen cultivars showed no detectable quantities of VOCs emission; in fact, the LipOXygenase pathway is activated when the fruit is damaged by the cut, producing a large quantity of volatile compounds, among which different C5 and C6 compounds, which are the main volatiles responsible for the positive aroma of olive oil (Angerosa *et al*., 2004). Meanwhile a great variability exists among the total VOCs emitted by the cut fruits of fifteen different cultivars (Table 2), ranging from 1061.8 ppbv in 'Ascolana Tenera' to 8767.5 ppbv for 'Carolea'. Therefore, the total emission from 'Carolea' was significantly higher compared to 'Ascolana Tenera'. The cultivars can be arbitrarily divided in three main groups according to their total VOCs emission: (a) low VOCs emission, including together with the cv. Ascolana tenera, Fasolona and

Table 2 - Analysis of variance (ANOVA) for the C5, C6, and total VOCs related to the fifteen cultivars

| Cultivar | C5 Compounds (ppbv) | C6 Compounds (ppbv) | Total VOCs (ppbv) | VOCs emission (Arbitrary ranking) |
|---|---|---|---|---|
| Ascolana tenera | 8.01±0.61 A | 180.73 ± 47.17 A | 1061.77 ± 120.13 A | low |
| Bianchera | 35.29±2.15 G | 1143.77 ± 148.60 F | 5101.34 ± 305.77 G | high |
| Carolea | 53.90±7.83 I | 2864.58 ± 543.60 I | 8767.54 ± 1024.09 G | high |
| Coratina | 12.02±1.15 D | 261.36 ± 34.62 ABC | 3177.13 ± 281.13 F | medium |
| Fasolona | 6.64±0.44 AB | 229.19 ± 35.91 AB | 1845.41 ± 124.20 DE | low |
| Frantoio | 11.86±1.43 D | 525.42 ± 96.22 DE | 2076.17 ± 149.65 ABC | medium |
| Itrana | 43.63±1.75 H | 2497.20 ± 222.50 H | 7877.17 ± 491.75 G | high |
| Leccino | 9.07± 0.51 BC | 264.10 ± 58.53 ABC | 3380.28 ± 148.72 F | medium |
| Maiatica di Ferrandina | 24.49±3.21 F | 1581.16 ± 262.15 G | 5339.86 ± 472.56 EF | high |
| Moraiolo | 17.41±1.52 E | 533.93 ± 67.89 DE | 2768.21 ± 150.04 BC | medium |
| Nocellara del Belice | 16.77±1.15 E | 544.07 ± 104.33 E | 2986.40 ± 128.63 D | medium |
| Palmarola | 9.01±0.57 B | 404.83 ± 64.22 CDE | 3142.02 ± 218.24 DE | medium |
| Sant'Agostino | 7.65±1.66 AB | 148.79 ± 27.75 A | 2309.40 ± 141.08 DE | medium |
| Santa Caterina | 8.73±1.61 B | 402.58 ± 55.28 CDE | 2121.11 ± 148.07 AB | medium |
| San Francesco | 11.54±0.76 CD | 368.17 ± 43.71 BCD | 1934.07 ± 65.61 C | low |

Different upper case letters within a column indicate the difference by the LSD test at the 99% confidence leve (p= 0.01).

San Francesco; (b) medium VOCs emission, including 'Leccino', 'Coratina', 'Palmarora', 'Nocellara del Belice', 'Moraiolo', 'Sant'Agostino', 'Santa Caterina', 'Frantoio' and (c) high VOCs emission, including 'Carolea', 'Itrana', 'Maiatica di Ferrandina', 'Bianchera'. Also C6 and C5 straight chain showed an ample variability; in particular, C6 compounds ranged from 148.79 ppbv for the cv. Sant'Agostino to 2864.58 ppbv for the cv. Carolea, with a fluctuation among the different cultivar of about 1:20. As the high amount of developed C6 compounds it is noteworthy to highlight the cv. Carolea, Coratina, and Itrana. On the other hand, the C5 compounds showed a minimum value of 6.64 ppbv (cv. Fasolona), and a maximum of 53.90 ppbv (cv. Carolea).

A biplot from Factor Analysis (Fig. 1) simultaneously represents the relationship between total VOCs, C6, and C5 compounds, highlighting the relative distances among the fifteen olive cultivars. The first axis explains the 97.25% of the total variability in the spectral data. The samples are clustered in two main groups (Fig. 1), characterized by higher VOCs emission (right quadrants), and lower VOCs emission (left quadrants). Although the different total amount, and despite the quantitatively different responses of the different cultivars, C5 and C6 compounds resulted directly related to the total VOCs emission (0.952 ppbv, and 0.964 ppbv, respectively), as a consequence of a more general phenomenon, the LipOXygenase (LOX) pathway, common to all the examined cultivars. Therefore, by evaluating the total VOCs amount, it is possible to analytically extrapolate the contribution of C5 and C6 in the total volatile compounds emission. Moreover, by the Factor Analysis plot it can be noted that some cultivars are characterized by higher C6 compounds emission (e.g. 'Itrana' and 'Maiatica di Ferrandina'), while other cultivars showed higher C5 emission (e.g. 'Bianchera' and 'Carolea').

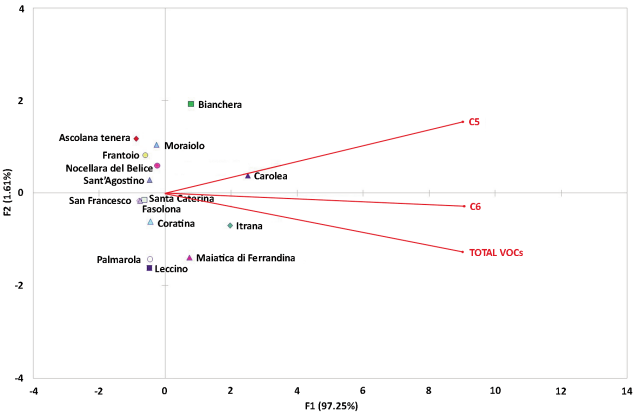
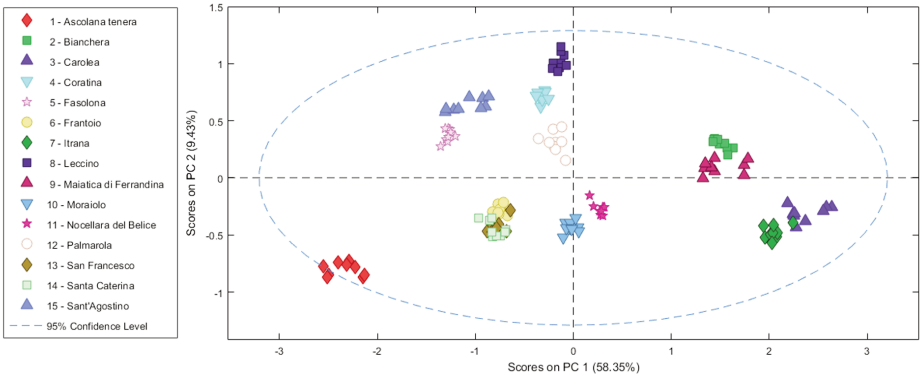



Fig. 1 - Biplot from Factor analysis. Relationships among the fifteen cultivars and the three groups of chemical compounds (C5, C6, total VOCs).

Subsequently a PCA (Fig. 2) was performed on the whole VOCs data set to (1) give a general overview of the cultivars ordination and (2) to detect if the cultivars can be grouped basing on their geographical origin and/or final product use. ANOVA results confirm (Table 2) that a high variability appears in the VOCs emission from the different cultivars, widely distributed without any particular tendency to clustering, excluding any possible link of the different cultivars due to their geographical origin or to the final product use. The first two components justified 58.32% for PC1 and 9.43% for PC2 of the total variability, respectively, indicating that the greatest amount of the total variance is explained by the PC1. The most important PC1 loadings resulted in eight *m/z* as reported in figure 2. In particular, *m/z* = 81.069 [Tentatively Identified (T.I.) as Hexenal fragment] and *m/z* = 99.080 (T.I. as 2,3-Hexenal) directly deriving from linolenic acid and responsible for the freshly cut grass odors, are previously reported as positive attributes in oils by Marone *et al.* (2017) and Taiti and Marone (2017).




| Protonated m/z | Chemical formula | PC 1 loadings | Min value | Max value |
|---|---|---|---|---|
| 53.038 | $C_4H_5^+$ | 0.22 | 10.02 | 172.29 |
| 55.054 | $C_4H_7^+$ | 0.22 | 52.68 | 1476.80 |
| 59.049 | $C_3H_7O^+$ | 0.22 | 8.16 | 173.27 |
| 65.038 | $C_5H_5^+$ | 0.21 | 0.94 | 23.79 |
| 71.086 | $C_5H_{11}^+$ | 0.21 | 0.00 | 13.63 |
| 79.054 | $C_6H_7^+$ | 0.23 | 16.20 | 364.47 |
| 81.069 | $C_6H_9^+$ | 0.28 | 81.83 | 3641.62 |
| 99.080 | $C_6H_{11}O^+$ | 0.24 | 5.12 | 116.01 |

Fig. 2 - Score plot from PCA related to the VOCs dataset (ppbv) for the fifteen cultivars; in the table the main PC1 loadings are reported.

The reported data confirmed the possibility to predict and quickly evaluate during all stages of the fruit ripening the presence of VOCs characterizing the positive flavors of the olive fruits obtained from defined cultivars or new breeding.

## 4. Conclusions

The data show a great variability in both amount and type of VOCs emitted by olive fruits, simultaneously collected in the same environment from fifteen different Italian cultivars. For example the total emission by 'Carolea' was significantly higher compared to 'Ascolana Tenera'. On the other hand, C5 and C6 compounds which most affect the aroma adding positive attributes to olive oil, were respectively 95% and 96% correlated to the total VOCs. Among the cultivars it is possible to note a different relationship between C5 and C6: the cv. Bianchera showed the highest C5/total VOCs ratio, while the cv. Itrana, and Carolea the highest C6/total VOCs ratio.

The presence of these two groups of compounds (C5 and C6) confirms that the cut of the fruit triggers the same phenomena that verifies at the moment of the olive processing, that is the activation of the LOX cascade, which gives rise to VOCs considered to be positive attributes for the olive oil aroma.

Further studies would be necessary to deepen and to develop the information that can be obtained from the use of the fruits to: (1) understand the behavior of the derived oils, and (2) to widen the genetic platform from which information can be acquired and (3) to start verifying the responses of fruits deriving from predetermined crosses. In conclusion, the possibility to early identify these compounds at the fruit level can allow to individuate the best conditions to produce high quality olive oils, and get useful information to individuate the best parents for the olive genetic improvement.

## Acknowledgements

The Authors would like to thank the Montepaldi Experimental farm of University of Florence for providing the samples used for these trials.

## References

ANGEROSA F., SERVILI M., SELVAGGINI R., TATICCHI A., ESPOSTO S., MONTEDORO G.F., 2004 - *Review: Volatile compounds in virgin olive oil: occurrence and their relationship with the quality*. - J. Chromatogr. A, 1054(1-2): 17-31.

FIORINO P., MARONE E., OTTANELLI A., 2010 - *Mechanical harvesting, productivity and superintensive planting systems in olive groves.* - Adv. Hort. Sci., 24(1): 91-94.

GARÇIA-VICO L., BELAJ A., SANCHEZ-ORTIZ A., MARTINEZ-RIVAS J.M., PEREZ A.G., SANZ C., 2017 - *Volatile Compound profiling by HS-SPME/GC-MS-FID of a core olive cultivar collection as a tool for aroma improvement of virgin olive oil*. - Molecules, 22(1): 141.

LINDINGER W., JORDAN A., 1998 - *Proton-transfer-reaction mass spectrometry (PTR-MS*): *On-line monitoring of volatile organic compounds at pptv levels*. - Chemical Society Reviews, 27: 347-375.

MARONE E., MASI E., TAITI C., PANDOLFI C., BAZIHIZINA N., AZZARELLO E., FIORINO P., MANCUSO S., 2017 - *Sensory, spectrometric (PTR-ToF-MS) and chemometric analyses to distinguish extra virgin from virgin olive oils*. - J. Food Sci. Technol., 54(6): 1368-1376.

MASI E., ROMANI A., PANDOLFI C., HEIMLER D., MANCUSO S., 2015 - *PTR-TOF-MS analysis of volatile compounds in olive fruits.* - J. Sci. Food Agric., 95(7): 1428-1434.

MAYR D., MARK T., LINDINGER W., BREVARD H., YERETZIAN C., 2003 - *Breath-by-breath analysis of banana aroma by proton transfer reaction mass spectrometry*. - Inter. J. Mass Spectrometry, 223: 743-756.

MORALES M.T., APARICIO R., CALVENTE J.J., 1996 - *Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil*. - Flavour Fragrance J., 11: 171-178.

MORALES M.T., RIOS J.J., APARICIO R., 1997 - *Changes in the volatile composition of virgin olive oil during oxidation: flavors and off-flavors*. - J. Agric. Food Chem., 45(7): 2666-2673.

TAITI C., COLZI I., AZZARELLO E., MANCUSO S., 2017 a - *Discovering a volatile organic compound fingerprinting of* Pouteria lucuma *fruits*. - Fruits, 72(3): 131-138.

TAITI C., MARONE E., 2017 - *EVOO or not EVOO? A new precise and simple analytical tool to discriminate virgin olive oils* - Adv. Hort. Sci, 31(4): 329-337.

TAITI C., MARONE E., LANZA M., AZZARELLO E., MASI E., PANDOLFI C., GIORDANI E., MANCUSO S., 2017 b - *Nashi or Williams pear fruits? Use of volatile organic compounds, physicochemical parameters, and sensory evaluation to understand the consumer's preference* - European Food Res. Techn., 243(11): 1917-1931.

UCEDA M., HERMOSO M., 1998 - *La calidad del aceite de oliva*, pp. 699-728. - In: BARRANCO D., R. FERNÁNDEZ-ESCOBAR, and L. RALLO (eds.) *El cultivo del olivo.* Mundi-Prensa, Madrid, Spain, pp.

ZUNIN P., BOGGIA R., SALVADEO P., EVANGELISTI F., 2005 - *Geographical traceability of West Liguria extravirgin olive oils by the analysis of volatile terpenoid hydrocarbons*. - J. Chromatography A, 1089(1-2): 243-249.

Adv. Hort. Sci., 2018 32(1): 149-153 DOI: 10.13128/ahs-22846

*short note*

AHS
Advances in Horticultural Science

# Monitoring in real time the changes in VOCs emission in sunflower and extra virgin olive oil upon heating by PTR-ToF-MS

**L. Sabbatini [1], C. Taiti [1], M. Redwan [1], E. Azzarello [1], E. Marone [2 (*)], S. Mancuso [1]**

[1] *Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università degli Studi di Firenze, Viale delle Idee, 30, 50019 Sesto Fiorentino (FI), Italy.*

[2] *Facoltà di Bioscienze e Tecnologie Agro-Alimentari e Ambientali, Università degli Studi di Teramo, Via R. Balzarini, 1, 64100 Teramo, Italy.*

*Key words:* Proton Transfer Reaction Time-of-Flight Mass Spectrometer, protonated masses, temperature, vegetable oils, Volatile Organic Compounds.

**(*) Corresponding author:**
emarone@unite.it

**Citation:**
SABBATINI L., TAITI C., REDWAN M., AZZARELLO E., MARONE E., MANCUSO S., 2018 - *Monitoring in real time the changes in VOCs emission in sunflower and extra virgin olive oil upon heating by PTR-ToF-MS.* - Adv. Hort. Sci., 32(1): 149-153



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 2 December 2017
Accepted for publication 23 March 2018

**Abstract**: **In this work the emission of volatile organic compounds (VOCs) upon the heating process of an extra virgin olive oil (EVOO) and a high oleic sunflower oil (SFO) was evaluated in real time by spectrometry. Two tests were carried out, in the first VOCs emitted from both kinds of oil were measured at room temperatures (not heated, NH) and at 180°C; in the second test, VOCs emission for selected masses were monitored under increasing temperatures over time: at room temperature not heated oils (NH), 60, 90, 120, 150, and 180°C, respectively. The spectra were acquired using a Proton Transfer Reaction Time of Flight Mass Spectrometer (PTR-ToF-MS). The total VOCs emission increased at 180°C, determined both by the rise of the amount of compounds present in the NH samples and by the formation of new masses generated by oxidative chemical reaction from triglycerides and fatty acids. From the set of results it is evident that a good control of the temperatures can be useful in reducing the quantities of masses potentially harmful to health in human food.**

## 1. Introduction

A common worldwide operation extensively used in food preparation is cooking by immersion in hot oil, as for fried and deep fried operations. Types of oils used for fried and deep frying operations vary with different cultures and are widely used both domestically and commercially. When frying and deep frying are used, fats and oils are carried at elevated tem-

peratures often over 180°C, which in presence of atmospheric oxygen (between 160 and 240°C) produce many flavor compounds (alkanes, fatty acids, aldehydes, ketones, polycyclic aromatic hydrocarbons), pleasant or unpleasant, and change their flavor stability. The flavor compounds released during the heating process are affected by some parameters such as cooking temperature, preparation time, chemical composition of the oils, physical and physicochemical constants and the presence of additives and contaminants (Fullana *et al.*, 2004). The volatile compounds and sensory characteristics from different edible oils (Brewer *et al*., 1999; Katragadda *et al*., 2010) were altered after intense heating and the main alterations were in the emission of volatile compounds linked to the oxidation process, compounds which are negatively related with the sensory attributes. In particular, the oxidation process in oils mainly lead to the formation of alkyl radicals, alkylperoxyl radicals and decomposition of hydroperoxide and cause the decay of both their nutritional and sensory quality (Velasco and Dobarganes, 2002; Silvagni *et al*., 2012). Moreover, there are some human health risks both linked to oil oxidations process and to many of the originated compounds released by the heating process (Vaclavik *et al*., 2013).

The PTR-ToF-MS tool represents a simple, and high-throughput strategy, applicable without any laborious sample preparations and treatments, particularly suited for analysis in real time of dynamic flavor release (both *in vitro* and *in vivo*) from diverse food matrices along the food-to-fork production chain (Costa *et al*., 2016).

The aims of this study are (1) to test the PTR-ToF-MS as a sampling system for the detection of the released compounds from not heated olive and sunflower oils and to compare with heated oils at their smoking point (180°C); (2) to monitor and evaluate in real-time the VOCs emitted from different oils, upon the heating from 25°C to 180°C, to understand the dynamic of oxidation phenomena.

## 2. Materials and Methods

*Vegetable oil material*

Two different types of commercial oils were used for this experiment, extra virgin olive oil (EVOO) and a high oleic sunflower oil (SFO). They have different heating temperatures, this latter (SFO) considered more resistant to thermal increases (Smith *et al*., 2007). Both oils were acquired at the local supermarket in Florence city (Italy). The analysis of volatile organic compounds (VOCs) emitted from both oils virgin has been obtained through a PTR-ToF-MS (Ionicon Analytik GmbH, Innsbruck, Austria), which is an instrument with high resolution mass and sensibility (5-10 ppt) and makes analysis possible without any pretreatment of samples. $H_3O^+$ was used as a reagent ion for the proton transfer reaction.

*Sample preparation and analysis*

*First experiment.* For this experiment, 100 ml of each type of oil was placed inside a 250 ml Pyrex glass jar, hermetically sealed; the jar's lid was equipped with two holes that permitted the insertion of Teflon tubes, which were respectively connected to a zero-air generator (Peak Scientific instruments, USA) and to the PTR-ToF-MS system. It was essential to avoid the entry of foreign substances inside the jar, and that was obtained by means of a sealing paste placed around the lid and the two entry holes. Oil samples were analyzed at two points: Room temperature (25°C) and at 180°C.

*Second experiment.* The same steps of the first experiment was followed, in addition each oil sample was heated by using a round electric heating plate and analyzed in real time to verify the VOCs emitted from both types of oil at different temperatures: room temperature (25°C), 60, 90, 120, 150, 180°C. Once reached each of the five thermal steps, the temperature was kept constant for 5 minutes and then passed to the subsequent thermal step. The evaluation of the aromatic profile of each oil examined was carried out in real time in order to monitor the behavior of the various volatile substances during the heating process. For each sample the analysis lasted for a period of about 50 minutes, recording a spectrum *per* second for a total of 60 spectra per minute. To determine the achievement of the selected temperatures, a trial was performed in parallel; in details, a thermometer was placed into the jar contained 100 ml of different kinds of oil and the times required to reach each temperature, for both samples, were measured as well. The whole analyses were carried out inside a conditioned room, with an internal temperature of 25°C in order not to condition the chemical reactions, as they are strongly sensitive to variations in temperature and humidity. The value of the white (control) represented by an empty sealant jar was recorded before starting with samples analysis, and the volatile profile of the white (control) has always been subtracted from the final value of

each sample.

The VOCs in the headspace were observed by direct injection into the PTR-ToF-MS and separation of single ions happened accordingly to their mass to charge (*m/z*) ratio. All the instrumental parameters were settled in the following way as in Taiti *et al.* (2017): drift tube ionization condition at 600 V and a continuous pressure of 2.20±0.02 mbar; while instrument internal calibration was based on: *m/z* = 29.997 ($NO^+$); *m/z* = 59.049 ($C_3H_7O^+$) and *m/z* = 21.022 ($H_3O^+$) and was performed off-line. The experiment data was acquired through the TofDaq software (Tofwerk AG, Switzerland) and all spectra were analyzed and acquired using a dead time of 20 ns for the Poisson correction and peak extraction following the methodology described in Cappellin *et al.* (2011), using a modified Gaussian peak shape.

## 3. Results

The signal intensity (ncps) generally vary among different oils. In figure 1 the signal intensity (ncps) of protonated *m/z* detected at 25°C and 180°C in sunflower oil (SFO) and extra virgin olive oil (EVOO) are clearly indicated. As a general consideration, the intensity of the signals is always higher in EVOO than in the SFO at the same temperature. At room temperature (25°C) the SFO spectrum is smoother, characterized by only 16 signals, being a very refined oil compared to EVOO. The EVOO presents instead 33 signals at room temperature when not heated, which are characteristics of the extra virgin olive oil in general, even if among them there are some masses that could generate off-flavor (*m/z* = 47.049, *m/z* = 61.028, *m/z* = 89.060) (Marone *et al.*, 2017; Taiti and Marone, 2017). At temperatures of 180°C, 43 *m/z* were measured in EVOO and 39 *m/z* in SFO, with the formation of 23 new masses generated as a result of the high temperature in SFO and 11 new *m/z* in EVOO, of which only 7 common to both matrices.

To better verify the intensity of the signals at temperatures below 180°C, some masses of particular relevance have been selected in SFO and EVOO, found in the most recent literature (Klein *et al.*, 2016). In particular, in the SFO emerge the masses *m/z* = 57, *m/z* = 101, and *m/z* = 113 (Fig. 2); these masses are also reported in Klein *et al.* (2016) as particularly relevant emissions by SFO. For olive oil emerge the masses *m/z* = 57, *m/z* = 101, *m/z* = 115, *m/z* = 143, also reported in Klein *et al.* (2016). Moreover, as its peculiar trend, it should be noted

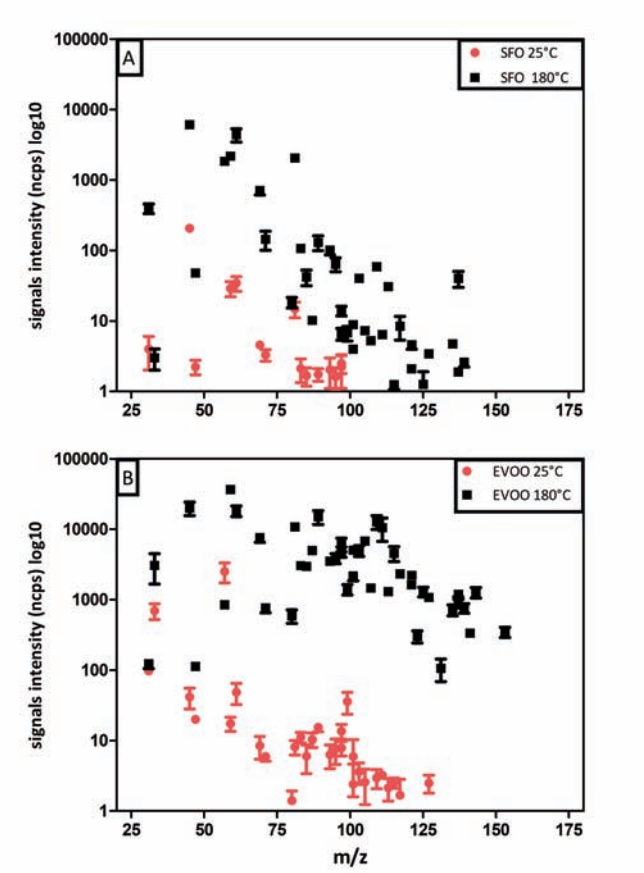



Fig. 1 - Signal intensity (ncps) of VOCs compounds in sunflower oil (SFO) and extra virgin olive oil (EVOO) at 25°C and 180°C. Bars represent standard deviations (n = 3).

the mass *m/z* = 45 (Fig. 2), that sharply increases in EVOO starting from 150°C.

The emission assessment of the selected masses by PTR-ToF-MS allows to directly examine in real time the trend of their quantities in SFO and EVOO in relation to the heating temperatures, avoiding alterations due to foods present together with the cooking oil, as generally found in the current scientific literature (Ontanón *et al.*, 2013; Klein *et al.*, 2016). The increasing temperature determines the increase of the emission of the total VOCs produced at each temperature point for both SFO and EVOO. The intensity of VOCs emission by the refined SFO, always appears lower than that of the EVOO. In both oils the increases are progressive, and related to the increasing temperature, in agreement with Katragadda *et al.* (2010). Both in SFO and EVOO the mass *m/z* = 57, still absent at 90°C, present at 120°C, showed a dramatic increase at 150°C according to Katragadda *et al.* (2010), and subsequently restart a trend proportional to the increase in temperatures. For each matrix all the other selected masses showed a common trend;

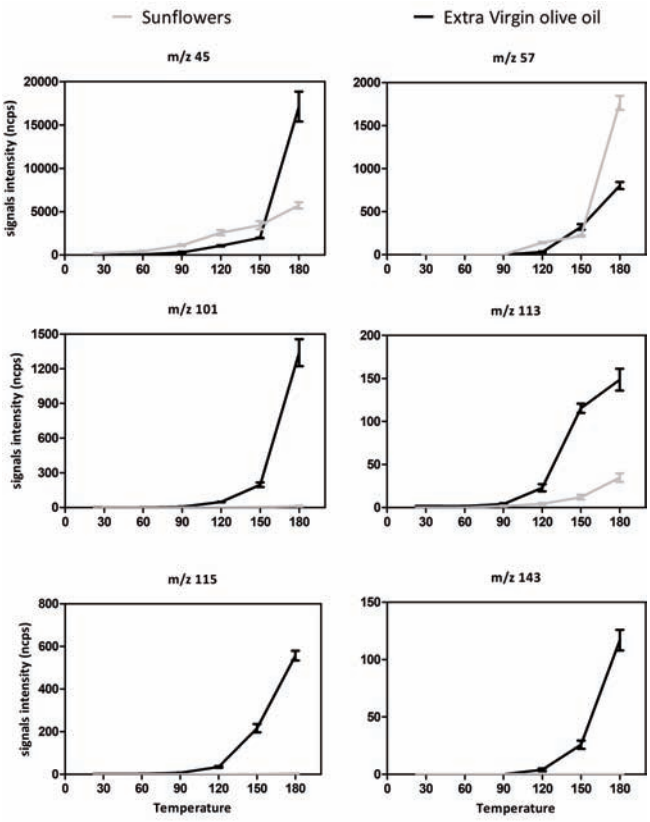



Fig. 2 - Trend of signal intensity (ncps) of different protonated m/z in sunflower oil (SFO) and extra virgin olive oil (EVOO) at different temperatures (25, 60, 90, 120, 150, and 180°C) upon heating

while they were absent, or present in small quantities, up to a temperature of 90°C, they have a relatively modest rise to 120°C, and showed a dramatic increase from 150°C, according to Nunes *et al*. (2013). The mass *m/z* = 57, always absent in the unheated oils, would derive from the dehydration of glycerol subjected to high temperatures (Klein *et al.,* 2016), and this would be a phenomenon common to all vegetable oils: the aldehydes that may be present in virgin olive oils as a result of a natural enzymatic oxidation of polyunsaturated fatty acids (LypOXigenase cascade, LOX) increase due to the temperature through a chemical oxidative reaction of the same polyunsaturated fatty acids (Muik *et al*., 2005).

## 4. Discussion and Conclusions

The PTR-ToF-MS tool represents a simple, and high-throughput strategy, applicable without any laborious sample preparations and treatments, for the real-time detection of oxidative changes occurred during the heating process of both vegetable oils. There are numerous studies that examine the quality of the oil, as well as its composition and characteristics during the heating or frying process; nevertheless, a fewer number of studies examine the composition of the fumes generated during this process.

In this work, it is pointed out that EVOO emissions are always higher than those of the SFO; the effect of the heating causes an increase in the emission of the masses present at the origin and the generation of new compounds, deriving from transformations of triglycerides and above all of the polyunsaturated fatty acids (Moreno *et al*., 1999). In particular, the trend of emissions in relation to the temperature has been highlighted for some selected masses, and it is clear that change due to chemical oxidation occur between 120 and 150°C, temperatures above which the production of volatiles increases enormously for all the examined masses. Since the increase in oil temperature proportionally increases the emission of VOCs, including some compounds that could be considered as pollutants, it is clear that an accurate control of the temperatures of oils used for frying food is important for the industry and for the home cooking. Many VOCs depend on the temperature, emissions at temperatures below the *smoke* point will be actually reduced. Some compounds, such as mass *m/z* = 57, start to form consistently already at relatively low temperatures (150°C). It should be noted that in the case of EVOO, masses of naturally occurring compounds are also very high, as they are derived from an oxidative process linked to enzymatic reactions of the polyunsaturated fatty acids (LipOXygenase pathway, LOX) (Campestre *et al*., 2017). The increase determined by high temperatures is no longer enzymatic but chemical oxidative. Further studies are needed to deepen the knowledge on the effect of heating temperature in the formation of compounds potentially harmful to human health, in order to reduce the effect of pollution not only at commercial but also at the domestic level, for the daily use of fried oils.

## References

BREWER M.S., VEGA J.D., PERKINS E.G., 1999 - *Volatile compounds and sensory characteristics of frying fats*. - J. Food Lipids, 6(1): 47-61.

CAPPELLIN L., BIASIOLI F., GRANITTO P.B., SCHUHFRIED E., SOUKOULIS C., COSTA F., TILLMAN M.D., GASPERI F., 2011 - *On data analysis in PTR-TOF-MS: from raw spec-*

*tra to data mining*. - Sens. Actuators B Chem., 155: 183-190.

CAMPESTRE C., ANGELINI G., GASBARRI C., ANGEROSA F., 2017 - *Review. The compounds responsible for the sensory profile in monovarietal virgin olive oils*. - Molecules, 22: 1833.

COSTA C., TAITI C., STRANO M.C., MORONE G., ANTONUCCI F., MANCUSO S., CLAPS S., PALLOTTINO F., SEPE L., BAZIHIZINA N., MENESATTI P., 2016 - *Multivariate approaches to electronic nose and PTR-ToF-MS technologies in agro-food products*, pp. 73-82. - In: RODRÍGEZ MENDÉZ M.L. (ed.) *Electronic noses and tongues in food science*. Elsevier Inc., Academic Press, Oxford, UK, pp. 309.

FULLANA A., CARBONELL-BARRACHINA A.A., SIDHU S., 2004 - *Comparison of volatile aldehydes present in the cooking fumes of extra virgin olive, olive, and canola oils.* - J. Agric. Food Chem., 52(16): 5207-5214.

KATRAGADDA H.R., FULLANA A., SIDHU S., CARBONELL-BARRACHINA A.A., 2010 - *Emissions of volatile aldehydes from heated cooking oils*. - Food Chemistry, 120: 59-65.

KLEIN F., PLATT S.M., FARREN N.J., DETOURNAY A., BRUNS E.A., BOZZETTI C., DAELLENBACH K.R., KILIC D., KUMAR N.K., PIEBER S.M., SLOWIK J.G., TEMIME-ROUSSEL B., MARCHAND N., HAMILTON J.F., BALTENSPERGER U., PRÉVÔT A.S.H., EL HADDAD I., 2016 - *Characterization of gas-phase organics using Proton Transfer Reaction Time-of-Flight Mass spectrometry: Cooking emissions*. - Environ. Sci. Technol., 50: 1243-1250.

MARONE E., MASI E., TAITI C., PANDOLFI C., BAZIHIZINA N., AZZARELLO E., FIORINO P., MANCUSO S., 2017 - *Sensory, spectrometric (PTR-ToF-MS) and chemometric analyses to distinguish extra virgin from virgin olive oils*. - J. Food Sci. Technol., 54(6): 1568-1376.

MORENO M.M., OLIVARES D.M., LOPEZ F.A., ADELANTADO J.G., REIG F.B., 1999 - *Analytical evaluation of polyunsaturated fatty acids degradation during thermal oxidation of edible oils by Fourier transform infrared spectroscopy.* - Talanta, 50(2): 269-275.

MUIK B., LENDL B., MOLINA-DÍAZ A., 2005 - *Direct monitoring of lipid oxidation in edible oils by Fourier transform Raman spectroscopy*. - Chemistry Physics lipids, 134(2): 173-182.

NUNES C.A., RIOS DE SOUZA V., CORRÊA S.C., DE CÁSSIA DA COSTA E SILVA M., CARVALHO BASTOS S., MARQUES PINHEIRO A.C., 2013 - *Heating on the volatile composition and sensory aspects of extra-virgin olive*. - OIL Ciênc. Agrotec., Lavras, 37(6): 566-572.

ONTANÓN I., CULLERÉ L., ZAPATA J., VILLANUEVA B., FERREIRA V., ESCUDERO A., 2013 - *Application of a new sampling device for determination of volatile compounds released during heating olive and sunflower oil: sensory evaluation of those identified compounds.* - Eur. Food Res. Technol., 236: 1031-1040.

SILVAGNI A., FRANCO L., BAGNO A., RASTRELLI F., 2012 - *Thermo-induced lipid oxidation of a culinary oil: The effect of materials used in common food processing on the evolution of oxidised species.* - Food chemistry, 133(3): 754-759.

SMITH S.A., KING R.E., MIN D.B., 2007 - *Oxidative and thermal stabilities of genetically modified high oleic sunflower oil*. - Food Chemistry, 102: 1208-1213.

TAITI C., MARONE E., 2017 - *EVOO or not EVOO? A new precise and simple analytical tool to discriminate virgin olive oils*. - Adv. Hort. Sci., 31(4): 329-337.

TAITI C., MARONE E., LANZA M., AZZARELLO E., MASI E., PANDOLFI C., GIORDANI E., MANCUSO S., 2017 - *Nashi or Williams pear fruits? Use of volatile organic compounds, physicochemical parameters, and sensory evaluation to understand the consumer's preference*. - European Food Res. Techn., 243(11): 1917-1931.

VACLAVIK L., BELKOVA B., REBLOVA Z., RIDDELLOVA K., HAJSLOVA J., 2013 - *Rapid monitoring of heat-accelerated reactions in vegetable oils using direct analysis in real time ionization coupled with high resolution mass spectrometry*. - Food Chem., 138(4): 2312-2320.

VELASCO J., DOBARGANES C., 2002 - *Oxidative stability of virgin olive oil*. - European J. Lipid Sci. Techn., 104(9-10): 661-676.