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CONTENTS

Ndereyimana A., Nyalala S., Murerwa P., Gaidashova S.
Growth, yield and fruit quality of tomato under different integrated management options against *Tuta absoluta* Meyrick 123

Rashidiani N., Nazari F., Javadi T., Samadi S.
Comparative postharvest responses of carnation and chrysanthemum to synthesized silver nanoparticles (AgNPs) 133

Amuji C.F., Beaumont L.J., Rodriguez M.E.
Simulating the impact of projected West African heatwaves and water stress on the physiology and yield of three tomato varieties 147

Menegatti R.D., Souza A.G., Bianchi V.J.
Different environments and doses of controlled-release fertilizer in peach rootstocks production 157

Rostami Tobnag G., Fattahi S., Khodabakhshzadeh A.
Effects of putrescine application in culture medium in improving chamomile [*Chamomilla recutita* (L.) Rauschert.] tolerance to osmotic stress under *in vitro* conditions 167

Raei Y., Sayyadi Ahmadabad M., Ghassemi-Golezani K., Ghassemi S.
Pinto bean and black mustard responses to bio-fertilizers under intercroppping system 175

De Paula V.F.S., Vilvert J.C., De Aráujo N.O., Do Nascimento I.B., De Medeiros J.F., Aroucha E.M.M.
Influence of plant biostimulant and spacing on production and postharvest conservation of watermelons cv. Quetzali 183

Abbasifar A., Valizadehkaji B., Karimi M., Bagheri H.
The first report: The effect of garlic extract on rooting of cuttings of some ornamental plants and fruit trees 191

Mirshekari A., Madani B., Wall M., Biggs A.R.
Aloe vera coatings maintain antioxidants of fig (*Ficus carica* L.) fruit during storage 205

Tadayon M.S., Hosseini S.M.
Effect of spread and shallow irrigation wetted area and application of organic mulch on citrus decline amelioration 213

Steidle neto A.J., Lopes D.C., Silva W.A.
Feasibility of Vis/NIR spectroscopy to detect and estimate fungicide residues on intact lettuces 223

Short Note

Fernandes V.N.A., Mangolin C.A., Neves A.F., Sousa Nogueira F.C., Zeni Neto H., Machado M.F.P.S.
Extraction of total protein from shoots of *Cereus* morphologicak variants (Cactaceae) for proteomic analysis 233

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Growth, yield and fruit quality of tomato under different integrated management options against *Tuta absoluta* Meyrick

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Key words: azadirachtin, *Beauveria bassiana,* integrated pest management, *Metarhizium anisopliae*, *Phytolacca dodecandra, Solanum lycopersicum* L., *Steinernema, Tephrosia vogelii.*

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The authors declare no competing interests.

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Abstract: This study evaluated the effect of entomopathogens and plant extracts, used against *Tuta absoluta*, on growth, yield, and fruit quality of tomato. Two field trials were carried out in a randomised compled block design, replicated thrice. The treatments were *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), Beauvitech®WP (*Beauveria bassiana*, Strain J25) as entomopathogens, *Tephrosia vogelii* and *Phytolacca dodecandra* as plant extracts, and azadirachtin 0.03% EC. Imidacloprid and water also were included as positive and negative controls, respectively. The best growth and yield parameters were recorded with the entomopathogens and azadirachtin, which were insignificantly different in most cases. The increase in yied of healthy fruit per plant (average of two trials) compared to the negative control (water spray) was 11.4, 10.8,10.1, 9.6, 3.96, 2.2, 11.7 and 2.4 folds for *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP, Beauvitech WP, *T. vogelii, P. dodecandra*, azadirachtin, and imidacloprid, respectively. There was no significant difference in number of leaves per plant and fruit quality parameters. The entomopathogens and azadirachtin, which exhibited a capacity to enhance tomato growth and reduced yield losses due to *T. absoluta*, are recommended to be included in integrated pest management programme on tomato.

1. Introduction

The increasing world population requires food security, which can be partly achieved by reducing the portion of food lost every year as a result of pests (Kumar and Omkar, 2018). However, yield losses inflicted by crop pests have been observed to increase constantly despite different strategies being implemented globally (Dhaliwal *et al*., 2010).

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegeta-

bles in the world and its fruits are a rich source of nutrients and health-promoting compounds (Luna-Guevara *et al*., 2014; Asensio *et al*., 2019). One average-sized tomato fruit offers 40% and 20% of the recommended daily amount of vitamins C and A, respectively. It also provides a significant amount of dietary fibres and minerals like calcium and potassium (Tigist *et al*., 2013). Furthermore, the antioxidant activity of ascorbic acid, carotenoids, and phenols protects humans against cancers and cardiovascular diseases (Tigist *et al*., 2013; Luna-Guevara *et al*., 2014). Therefore, any technology used on tomato crop has to be investigated not only for its effect on growth and yield but also on fruit quality parameters.

Several pests have been reported to attack tomato throughout its production cycle (Kumar and Omkar, 2018). The tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), was recognised among the major pests since 1964 in Argentina from where it invaded the rest of South America (Desneux *et al*., 2010). Following Spain invasion during the year 2006, the pest spread to many other European countries, the Middle East, more than 40 African countries, and almost all Southern West and Central Asian countries neighbouring China, the world's largest tomato producer (Biondi *et al*., 2018; Mansour *et al*., 2018). In only one decade, *T. absoluta* spread drastically and the world tomato production area under its invasion increased from 3% to 60% (Biondi *et al*., 2018). In Rwanda, *T. absoluta* was first recorded in Bugesera District in 2015 (FAO, 2015), after which it quickly spread in all tomato production areas of the country. The damage inflicted by *T. absoluta* affects negatively its growth and development and can lead to total crop failure (Desneux *et al*., 2010; Biondi *et al*., 2018). This calls for concerted efforts from different stakeholders in developing effective management strategies against this devastating pest.

Synthetic pesticides have been observed to be less effective against *T. absoluta* (Roditakis *et al*., 2013) and are associated with various challenges and harmful effects (Brahman *et al*., 2012; Kumar and Omkar, 2018). The concept of integrated pest management (IPM) was developed to address the drawbacks of solely relying on chemical control. In this perspective, alternatives to synthetic insecticides with reduced negative effects have been the object of research in several parts of the world (Biondi *et al*., 2018). A lot has been done on natural enemies, which are used in biological control of *T. absoluta* in some parts of the world (Desneux *et al*., 2010; El-Ghany *et al.*, 2016; Giorgini *et al*., 2019). Different biopesticides based on entomopathogens and botanical insecticides have also been evaluated and shown to be effective against this pest. However, these studies have been limited to specific biocontrol strains/species and also have been carried out mainly in the pest's area of origin (Jallow *et al*., 2019). Besides, many other studies have been limited to laboratory conditions (Youssef, 2015; El-Ghany *et al.,* 2016; Giorgini *et al*., 2019). There is also a scarce information on the effects on different *T. absoluta* management options on growth, yield and fruit quality of tomato.

Entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPFs) and plant extracts (PEs) are among the claimed options for effective management of *T. absoluta* (Mansour *et al*., 2018). Laboratory studies in Rwanda recommended some EPNs, EPFs, and PEs which can be advanced to field evaluation stage (Ndereyimana *et al*., 2019 a, b, c). To this aim, the current study investigated the growth, yield and fruit quality of tomato as affected by entomopathogens and plant extracts against *T. absoluta*.

2. Materials and Methods

Study site

This study was carried out in Bugesera District of Rwanda, in a farmer's field located at 02° 32' 355" South latitude, 30° 26' 963" East longitude and an elevation of 1338 m above sea level. The average annual rainfall and temperature are 854 mm and 21.4°C, respectively (Kabirigi *et al.,* 2017).

Experimental design, trial establishment, and treatments application

The study evaluated nine treatments in a randomised complete block design with three replications. The individual experimental plots were 3 m long and 2 m wide, with 1.5 m wide paths between them. Thirty days old, healthy and uniform tomato cv. Roma seedlings were transplanted into the plots applied with 20 t of organic manure per hectare and mulched with dry grass. Transplanting for trials one and two was carried out on 3rd April 2019 and 28th June 2019, respectively.

The treatments included: two local EPN isolates (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2b-1), two commercial formulations of EPFs [Metatech® WP: *Metarhizium anisopliae* (Metsch.) Sorok, Strain FCM Ar 23B3, 5 x 109 CFUs/g,

and Beauvitech® WP: *Beauveria bassiana* (Bals.) Vuill., Strain J25, 1 x 1010 CFUs/g], two local plant extracts (*Tephrosia vogelii* and *Phytolacca dodecandra*), azadirachtin 0.03% EC (Nimbecidine), imidacloprid (Confidor SL 200) and water. The two last treatments were included as positive and negative controls, respectively. The two EPN isolates used were obtained from Biological Control Laboratory - EPN Production Facility at Rwanda Agriculture and Animal Resources Development Board (RAB) (Yan *et al*., 2016). Mass production of the EPNs was done through *in-vivo* method using *Galleria mellonella* larvae (Kaya and Stock, 1997). For field applications, these EPNs were formulated into sponges and were used at a concentration of 5 x 10^9 IJs/ha (Gözel and Kasap, 2015).

The EPF formulations were obtained from Dudutech Division, Flamingo Horticulture (K) Ltd, Naivasha, Kenya and were used at a concentration of 250 g/ha. The two local plant extracts were prepared from leaves of local plants (*T. vogelii* and *P. dodecandra*). The fine powder was obtained (using an electric grinder) from the leaves dried in a shaded area, mixed with boiled water and kept for 12 hours. The concentration used for field application was 15% weight/volume (w/v) and filtration was done using a muslin cloth. Azadirachtin 0.03% EC (Nimbecidine) and imidacloprid (Confidor SL 200) were used at the rates of 5 ml and 1 ml, respectively, per litre of water. All these treatments were applied weekly using a knapsack sprayer and the application volume was 1000 l/ha (Brusselman *et al*., 2012).

Cultural operations

Apart from the difference in applied treatments, all other cultural operations were uniformly done in all the experimental plots. Fungicide application was done every week by alternating Copper oxychloride 50% WP with fungicides containing Mancozeb 80% or Mancozeb (640 g/kg) + Metalaxyl (80 g/kg). Each tomato plant was fertilised with 10 g of NPK 17-17-17 as basal fertiliser, supplemented with 4 g of Urea 46% on 30th day after transplanting as per RAB recommendation. Other cultural practices like watering, weeding, and pruning were carried out conventionally.

Data collection and analysis

Data were collected on growth, yield, and fruit quality parameters. Plant growth parameters: plant height, stem diameter and number of leaves per plant, were recorded every two weeks. Plant height (cm) was measured from the ground to the tip of each of five randomly selected plants using a metre tape. Stem diameter (mm) was measured from the collar using a digital vernier caliper. The number of leaves arising from the main stem was counted. For yield parameters, the numbers of flower trusses per plant and flowers per truss were recorded 40 days after transplanting, while the number of fruits per truss was recorded 60 days after transplanting. The number and yields of healthy and bored fruits were recorded during the harvesting period, which started 72 and 70 days after transplanting in trials one and two, respectively. All the above parameters were taken from five plants selected randomly in the middle of each plot.

Fruit quality parameters, namely fruit firmness (Kg F/cm^2), total soluble solids (TSS) (°Brix), beta-carotene (mg/100 g of fruit), lycopene (mg/100 g of fruit), and ascorbic acid (mg/100 g of fruit), were recorded. To determine fruit firmness, tomatoes were harvested at the pink stage and stored at room temperature until the uniform red ripe stage. Then, five fruits were randomly selected from each treatment lot and fruit firmness measured in the equatorial zone of each tomato using a penetrometer (Ritenour *et al*., 2002). Total soluble solids were determined on the same fruits used for the determination of fruit firmness using a refractometer (RHW Refractometer, Optoelectronic Technology Company Limited, UK) (Majidi *et al*., 2011). Beta-Carotene was obtained following the method described by Delia *et al*. (2004). Lycopene was extracted using acetone and analysed in a spectrophotometer at 503 nm. Lycopene content was then calculated using the formula given by Ranganna (1997) as follows:

$$\text{Lycopene content} = 3.1206 \times A \times V \times D \times \frac{100}{(W \times 100)}$$

where A = Absorption, V = Volume made up, D = Dilution, W = Weight of Sample. Ascorbic acid was determined by titration with 2,6-dichlorophenolindophenol dye (AOAC, 1990).

The distribution of the collected data was assessed and the appropriate transformation was undertaken, where necessary, before subjecting them to analysis of variance. In both trials, the numbers of healthy and bored fruits per plant were square-root transformed, while the yield of healthy and bored fruits per plant were log-transformed. The number of fruits per truss was log-transformed in trial one, and arcsine-transformed in trial two; while the number of flowers per truss was arcsine-transformed in trial two. All other parameters were

analysed without transformation. To determine the effect of the treatments on tomato fruits yield and quality, analysis of variance was carried out; and the means for significantly different treatments (at P≤0.05) were separated using Tukey's honestly significant difference test. The data analysis was carried out using the Statistical Analysis System package, SAS software version 9.2 (SAS Institute, 2010).

3. Results

Tomato growth parameters

Plant height was significantly (P≤0.05) influenced by the studied treatments from 30 days after transplanting (DAP) (Fig. 1). In both trials, the plant height was not significantly different at 15 DAT; with an average of 14.9 and 15.3 cm for trials one and two, respectively. Plant height increased with time but became almost constant at 45 DAT. In trial one, there was no significant difference among the entomopathogens (EPNs and EPFs) and azadirachtin on all days of observation. *Tephrosia vogelii* was not significantly different from all the above at 30, 45, and 60 DAT, except *Steinernema* sp. RW14-M-C2a-3. Lower plant height was recorded with *P. dodecandra* and the controls, which were insignificantly different. In trial two, plant height did not significantly differ among the treatments, except *P. dodecandra* and the controls which had lower plant height than others.

Stem diameter did not significantly differ among the treatments at 15 and 30 days after transplanting (DAT) in trial one and at 15 DAT in trial two (Fig. 2). In addition, only the stem diameter in the negative control was significantly lower as compared to the other treatments at 45 DAT in trial one. *Phytolacca dodecandra* and imidacloprid were similar to the negative control, with significantly lower stem diameter (P≤0.05) compared to the other treatments at 60 DAT. For trial two, *P. dodecandra* and negative control had significantly lower stem diameter as compared to the other treatments at 30 DAT; but at 60 DAT it was only the negative control which had significantly lower stem diameter as compared to azadirachtin and all entomopathogens except Beauvitech® WP.

The number of leaves per plant was not significantly affected by the evaluated treatments in both

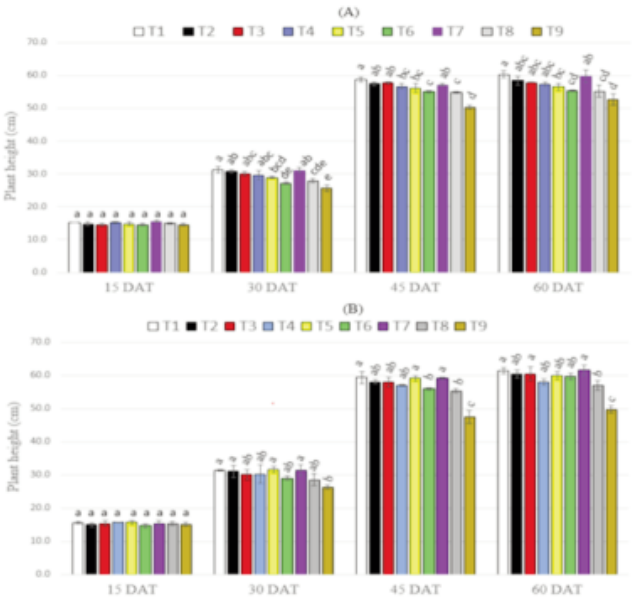

Fig. 1 - Plant height of tomato cv. Roma under different treatments against *Tuta absoluta* in trials one (A) and two (B). T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii*, T6: *Phytolacca dodecandra*, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: Water; DAT: Days after transplanting; Different letters above the bars indicate significant difference according to Tukey's test (P≤0.05).

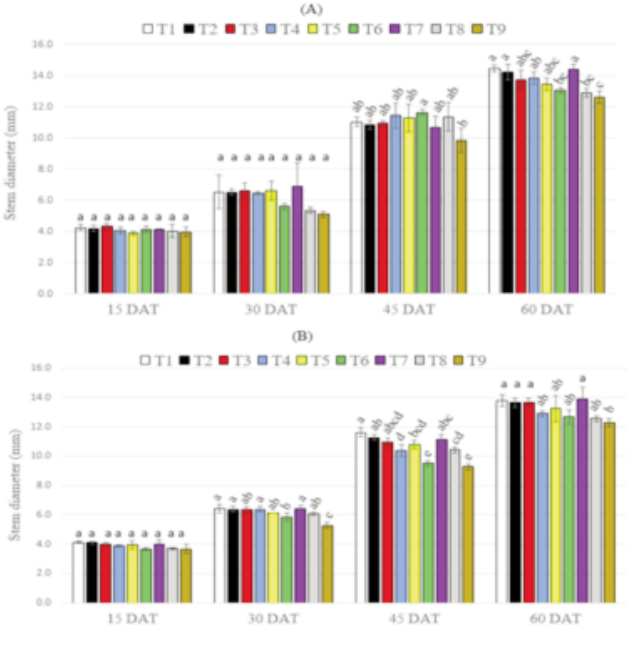

Fig. 2 - Stem diameter of tomato cv. Roma under different treatments against *Tuta absoluta* in trials one (A) and two (B). T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii*, T6: *Phytolacca dodecandra*, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: Water; DAT: Days after transplanting; Different letters above the bars indicate significant difference according to Tukey's test (p≤0.05).

trials. However, the general trend observed in both trials was that slightly higher (but not significantly different) number could be obtained in plots treated with Metatech® WP (*M. anisopliae*, Strain FCM Ar 23B3) and azadirachtin in trial one; and with *Steinernema* sp RW14-M-C2a-3 and Beauvitech® WP (*B. bassiana*, Strain J25) in trial two (Fig. 3). The average numbers of leaves per plant recorded at 60 DAT in trial one were 13.0, 12.8,14.0, 12.5, 12.8, 12.6, 13.3, 11.9, and 12.6; while in trial two they were 11.8, 11.6, 11.7, 11.9, 11.1, 11.1, 11.5, 11.2, and 11.3, in plots treated with *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2b-1, Metatech®WP (*M. anisopliae*, Strain FCM Ar 23B3), Beauvitech® WP (*B. bassiana*, Strain J25), *T. vogelii, P. dodecandra*, azadirachtin, imidacloprid, and water, respectively.

Effect of entomopathogens and plant extracts on tomato yield

The evaluated treatments significantly (P<0.001) influenced tomato yield parameters in both trials (Table 1). Generally, plots treated with the entomopathogens or azadirachtin had higher performance as compared to those with plant extracts or controls. A similar number of flower trusses per plant was recorded by *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP,

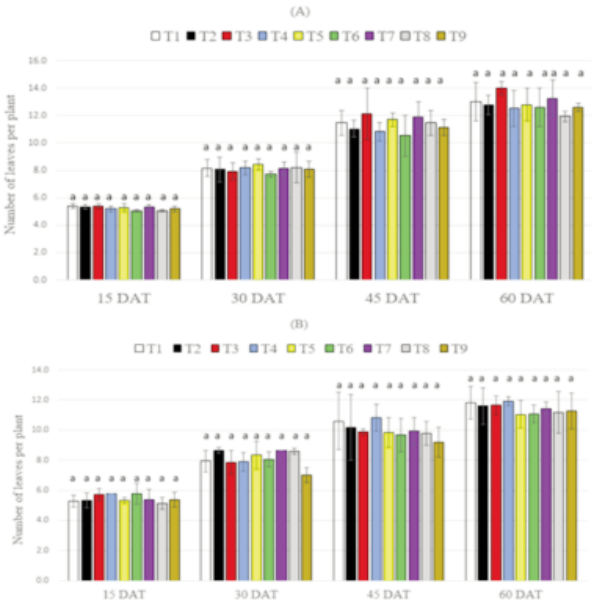

Fig. 3 - Number of leaves per plant for tomato cv. Roma under different treatment against *Tuta absoluta* in trials one (A) and two (B). T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii*, T6: *Phytolacca dodecandra*, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: Water; DAT: Days after transplanting; Similar letters above the bars indicate non-significant difference according to Tukey's test (P≤0.05).

Table 1 - Yield parameters (mean ± SD) of tomato under different entomopathogens and plant extracts treatments

Treatments	Number of flower trusses/plant	Number of flowers/truss	Number of fruits/truss	Number of healthy fruits/plant	Number of bored fruits/plant	Yield of healthy fruits (g/plant)	Yield of bored fruits (g/plant)
Trial one							
T1	11.9 ± 0.2 a	9.7 ± 0.4 a	4.1 ± 0.3 a	5.9 ± 0.2 a	5.8 ± 0.2 a	406.3 ± 10.9 a	333.3 ± 33.4 a
T2	11.6 ± 0.4 abc	8.5 ± 0.1 bc	4.0 ± 0.1 a	5.6 ± 0.2 a	5.1 ± 0.4 a	381.0 ± 22.3 a	286.8 ± 8.8 a
T3	11.7 ± 0.2 ab	7.7 ± 0.2 cd	4.1 ± 0.1 a	5.5 ± 0.3 a	6.1 ± 0.4 a	374.4 ± 23.5 a	328.8 ± 34.9 a
T4	11.4 ± 0.1 abc	7.3 ± 0.3 de	3.8 ± 0.5 a	5.4 ± 0.2 a	5.6 ± 0.4 a	335.0 ± 34.5 a	313.9 ± 17.3 a
T5	10.9 ± 0.2 bc	7.4 ± 0.1 de	3.0 ± 0.3 b	2.5 ± 0.3 b	3.2 ± 0.2 b	151.0 ± 12.3 b	161.7 ± 9.7 b
T6	10.8 ± 0.3 c	6.4 ± 0.2 ef	2.9 ± 0.2 b	1.5 ± 0.1 c	2.6 ± 0.2 cb	81.5 ± 12.5 b	126.2 ± 10.1 b
T7	12.7 ± 0.1 a	9.4 ± 0.6 ab	4.3 ± 0.2 a	6.5 ± 0.2 a	4.9 ± 0.6 a	402.9 ± 12.7 a	275.7 ± 29.5 a
T8	10.8 ± 0.3 c	6.5 ± 0.3 ef	3.0 ± 0.4 b	1.7 ± 0.2 c	2.3 ± 0.5 cb	86.6 ± 9.0 b	109.9 ± 26.2 bc
T9	10.8 ± 0.5 c	5.6 ± 0.7 f	2.5 ± 0.3 b	0.7 ± 0.4 d	1.9 ± 0.5 c	32.5 ± 8.2 c	83.7 ± 22.1 2 b
CV	2.5	4.89	6.53	4.39	5.76	4.2	2.23
P	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Trial two							
T1	9.0 ± 0.2 a	7.7 ± 0.3 a	3.8 ± 0.3 a	5.5 ± 0.2 a	4.6 ± 0.7 a	367.8 ± 5.2 a	249.5 ± 22.1 a
T2	9.0 ± 0.2 a	7.4 ± 0.2 a	3.7 ± 0.2 a	5.4 ± 0.2 a	4.7 ± 1.0 a	350.9 ± 12.1 a	255.9 ± 38.1 a
T3	8.8 ± 0.2 ab	5.6 ± 0.2 cb	3.8 ± 0.4 a	4.9 ± 0.4 a	5.6 ± 0.8 a	309.7 ± 26.3 a	302.4 ± 45.9 a
T4	8.8 ± 0.5 ab	5.5 ± 0.2 cb	3.6 ± 0.2 a	4.9 ± 0.4 a	5.3 ± 1.0 a	319.4 ± 33.5 a	273.6 ± 64.8 a
T5	8.8 ± 0.2 ab	5.9 ± 0.2 b	2.8 ± 0.1 b	2.0 ± 0.4 b	4.4 ± 0.4 a	113.1 ± 13.4 b	220.9 ± 18.2 a
T6	8.1 ± 0.2 b	5.2 ± 0.3 cb	2.4 ± 0.4 b	1.4 ± 0.2 b	2.1 ± 0.1 b	67.0 ± 8.5 c	114.8 ± 17.0 b
T7	9.3 ± 0.2 a	8.0 ± 0.3 a	4.0 ± 0.2 a	6.0 ± 0.4 a	4.7 ± 0.1 a	392.5 ± 38.1 a	266.6 ± 19.6 a
T8	8.7 ± 0.1 ab	5.5 ± 0.3 cb	2.7 ± 0.2 b	1.6 ± 0.3 b	1.9 ± 0.5 b	74.7 ± 8.1 c	96.7 ± 22.5 b
T9	8.1 ± 0.2 b	5.0 ± 0.2 c	2.2 ± 0.3 b	0.8 ± 0.2 c	1.7 ± 0.2 b	35.7 ± 6.7 d	80.0 ± 10.10 b
CV	3.02	2.31	4.17	5.11	7.94	2.3	3.05
P	0.0004	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

T1: *Steinernema* sp. RW14-M-C2a-3, T2: *Steinernema* sp. RW14-M-C2b-1, T3: Metatech®WP (*Metarhizium anisopliae*, Strain FCM Ar 23B3), T4: Beauvitech® WP (*Beauveria bassiana*, Strain J25), T5: *Tephrosia vogelii, T6: Phytolacca dodecandra*, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: Water. Means followed by the same letter (s) are not significantly different (Tukey's test, P≤0.05)

Beauvitech WP, and azadirachtin. These values were significantly (P<0.001) higher than *T. vogelii*, *P. dodecandra*, imidacloprid, and water spray in trial one. In trial two, the effect of *T. vogelii* and imidacloprid was similar to all the treatments but the plot treated with EPNs and azadirachtin recorded a significantly higher number of flower trusses per plant than the negative control. The number of flowers per truss was significantly higher with *Steinernema* sp. RW14-M-C2a-3 and azadirachtin in trial one, and with all entomopathogenic nematodes and azadirachtin in trial two. Higher numbers of fruits per truss, healthy and bored fruits per plant were recorded with all entomopathogens and azadirachtin, in both trials. A similar trend was observed in the yield of healthy and bored fruits per plant.

Effect of entomopathogens and plant extracts on tomato fruit quality

Tomato fruit quality parameters were not significantly influenced by the applied treatments against *T. absoluta*. The results obtained were so close to each other that it is not easy to find any trend amongst the treatments (Fig. 4). The overall average values obtained were 3.2 and 3.3 kg F/cm^2 for fruit firmness, 4.2 and 4.4°Brix for TSS, 8.3 and 8.1 mg/100 g of fruit for beta-carotene, 5.4 and 5.5 mg/100 g of fruit for lycopene, 14.36 and 14.6 mg/100 g of fruit for ascorbic acid in trials one and two, respectively.

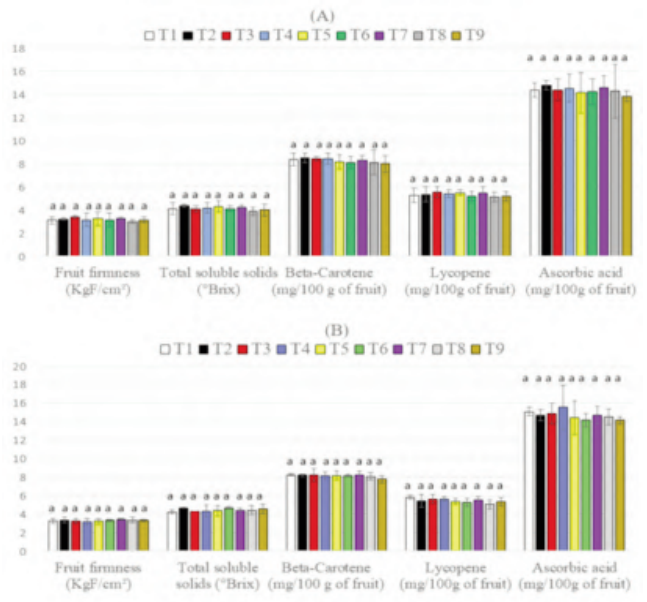

Fig. 4 - Fruit quality parameters of tomato cv. Roma under different treatment against Tuta absoluta in trials one (A) and two (B). T1: Steinernema sp. RW14-M-C2a-3, T2: Steinernema sp. RW14-M-C2b-1, T3: Metatech®WP (Metarhizium anisopliae, Strain FCM Ar 23B3), T4: Beauvitech® WP (Beauveria bassiana, Strain J25), T5: Tephrosia vogelii, T6: Phytolacca dodecandra, T7: azadirachtin 0.03% EC, T8: imidacloprid, T9: Water; Similar letters above the bars indicate non-significant difference according to Tukey's test (P≤0.05).

4. Discussion and Conclusions

Scarce studies have been conducted on the effects of entomopathogens and plant extracts on growth, yield and fruit quality of tomato. The significant differences observed in plant height and stem diameter could be due to the differences in the efficacy of studied treatments against *T. absoluta*. The damages inflicted by *T. absoluta* larvae my have affected the physiological and biochemical reactions of tomato plants, so that plant growth was consequently affected (Desneux *et al*., 2010). *Beauveria bassiana* which was reported to exhibit endophytic activity by colonising vascular tissues would be expected to impair the normal plant growth. However, different researchers reported that *B. bassiana* does not impede tomato growth (Klieber and Reineke, 2016; Allegrucci *et al*., 2017). On the other hand, since *T. vogelii* is a rich source of nitrogen, fixed through biological nitrogen fixation (Stevenson *et al*., 2012), more growth would be expected in this treatment compared to the others because nitrogen is more involved in plant growth and biomass production (Larbat *et al*., 2016). This was, however, not observed in this study and could be explained by the fact that the amount sprayed as an insecticide was too little to have a direct significant effect on plant growth. Finally, the insignificant difference in the number of leaves per plant despite the treatments could be because this parameter is associated with the genetic makeup of the plant (Kaushik *et al*., 2011) and not with cultural practices including pest management.

The significant difference in flower-related parameters could also be due to the difference in the efficacy of the studied treatments. By attacking the floral parts, *T. absoluta* larvae might have damaged some of them before they differentiate into flowers and caused others to drop; which could be the explanation for the flower abortion observed in this study. These results are in agreement with Cherif *et al*. (2013) who reported that *T. absoluta* larvae can damage tomato flower parts and cause flower drop.

The observed significant difference in yield parameters may also have arisen from the indirect effect of *T. absoluta* larvae through their feeding

activity in leaf mesophyll (Biondi *et al.*, 2018), which might have slowed down the process of assimilates synthesis and partitioning for their utilisation by different plant organs, including flower parts and fruits. In agreement with the above observation, Desneux *et al*. (2010) and El-Ghany *et al.* (2016) also reported that a tomato attack by *T. absoluta* disturbs its normal growth, development and the subsequent yield. Thus, higher numbers of flower trusses per plant, flowers per truss and fruits per truss recorded with entomopathogens and azadirachtin suggest that these treatments can reduce tomato yield loss as compared to the plant extracts and the controls (imidacloprid and water spray).

In their study, Rab and Haq (2012) found that the number of flowers per truss varied from 17.1 to 30.8 while the number of fruits per cluster was 4.1-6.4 for tomato cv. Roma. However, in the present study, a range of 5.6-9.7 flowers per truss and 2.2-4.3 fruits per truss was obtained. This indicates the ability of *T. absoluta* to negatively affect the flower and fruit-bearing capacity of tomato plant. This is one of the reasons for high yield losses frequently observed with *T. absoluta* infestations (Cherif *et al*., 2013; Biondi *et al*., 2018).

The higher numbers and yield of healthy fruits that were obtained with EPNs, EPFs, and azadirachtin support our earlier findings in laboratory experiments (Ndereyimana *et al*., 2019 a, b, c). In line with the findings of this study, Braham *et al*. (2012), Gözel and Kasap (2015), Youssef (2015), and El-Ghany *et al.* (2016) reported that EPNs, EPFs, and azadirachtin result in better control of *T. absoluta*. The performance of plant extracts and imidacloprid (positive control) remained low as it was in our previous laboratory studies (Ndereyimana *et al*., 2019 a, b). Negative control also recorded very low yield, which was consistent with Desneux *et al*. (2011) and Biondi *et al*. (2018) who emphasized that if there are no serious pest management strategies that are meticulously implemented, the yield loss might reach 100%.

Higher number and yield of bored fruits obtained from plots treated with entomopathogens and plant extracts, as compared to plant extracts and controls, might have resulted from the reduced number of aborted and damaged flowers by *T. absoluta* in the plots where these treatments were applied. Although these fruits survived from early abortion and the dropping of progenitor flowers, they were more exposed to *T. absoluta* because they were many, and thus a group of them was later bored by the pest that spoiled their quality. Compared to the negative control, the yield of healthy fruits obtained with *Steinernema* sp. RW14-M-C2a-3, *Steinernema* sp. RW14-M-C2a-3, Metatech®WP, Beauvitech®WP, and azadirachtin increased 12.5, 11.7, 11.5, 10.3 and 12.4 folds, respectively. While compared to the positive control, it was 4.8, 4.5, 4.2, 4.1 and 5.0 folds, respectively. This confirms that, despite the invasive nature of *T. absoluta*, different management options can reduce significantly its negative impact on the crop. However, dependence on synthetic insecticides should be discouraged as evidenced by the results of this study, which are consistent with several other researchers (Desneux *et al*., 2010; Roditakis *et al*., 2013; Biondi *et al*., 2018).

The commercial value of bored fruits is lost because they are not preferred by customers as external appearance and absence of defects are among the factors determining consumer preference (Asensio *et al.,* 2019). In addition to the larvae that enter inside the fruits, also some pathogens like fungi often get inside through the created holes and cause fruit decaying before or after harvest (Desneux *et al*., 2010). The findings of this study are supported by previous researchers who worked on other pests and reported that crop pests are among the main factors reducing the yield and quality of field horticultural produce by direct feeding or by favouring several diseases (Kumar and Omkar, 2018). Thus, implementation of IPM is worth to ensure better yield and quality of tomato crop.

Since the damage inflicted by *Tuta absoluta* on the leaves of tomato plants negatively affects its physiological processes (Desneux *et al*., 2010; Biondi *et al*., 2018) and fruit total soluble solids are translocated from the photosynthetic activities in the leaves (Beckles, 2012), significant difference in fruit quality parameters was expected among treatments with different *T. absoluta* infestation levels. Similarly, the entomopathogens and azadirachtin that exhibited better *T. absoluta* control would have also resulted in higher quality fruits as compared to the plant extracts and the controls´ treatments. The observed non-significant difference in tomato fruit quality parameters: firmness, total soluble solids, beta-carotene, lycopene, and ascorbic acid among treatments against *T. absoluta*, therefore may be attributed to other factors such as variety, crop nutrition, climatic conditions, fruit ripening stage, and storage period (Marsic *et al*., 2011; Rab and

Haq, 2012; Tigist *et al.*, 2013; Asensio *et al*., 2019).

Fruit firmness results obtained in this study fall in the range of the values obtained by Rab and Haq (2012). Fruit firmness is an important quality parameter that determines fruit shelf-life and resistance to mechanical damage (Tigist *et al*., 2013). In line with the current study, Parmar *et al.* (2018) also obtained a TSS value of 4.8 °Brix for tomato cv. Roma under organic management system. Also, TSS values obtained by Rab and Haq (2012) ranged from 4.08 to 6.10 °Brix under different rates of calcium chloride and borax. The values of beta-carotene and lycopene recorded in this study are close to what was obtained by Parmar *et al.* (2018) (8.34 mg/100 g and 5.38 mg/100 g of fruit, respectively) for the same variety (Roma) produced organically. The ascorbic acid results obtained in this study agree with the earlier findings of Tigist *et al*. (2013) who obtained the values of 13.2 and 14.8 mg/100 g after four and eight days of room temperature storage, respectively, for Tomato cv. Roma fruits harvested at the green mature stage.

According to Tigist *et al*. (2013), these quality parameters develop into fruit during the pre-harvest period and they do not get improved after harvesting. However, they can be maintained by proper post-harvest handling and storage. Since pre-harvest activities are responsible for the development of quality parameters in tomato fruits, any technology used to improve its production should also be assessed for its effect on fruit quality.

As a conclusion, the studied entomopathogens and plant extracts significantly affected tomato growth and yield but not the fruit quality parameters. Better yield performance can be obtained with the entomopathogenic nematode isolates (*Steinernema* sp. RW14-M-C2a-3 and *Steinernema* sp. RW14-M-C2a-3), commercial formulations of entomopathogenic fungi (Metatech®WP: *Metarhizium anisopliae*, Strain FCM Ar 23B3 and Beauvitech®WP: *Beauveria bassiana*, Strain J25) and azadirachtin 0.03% EC, which were not significantly different. These biorational control agents are recommended to be included in the IPM of *Tuta absoluta*. The results of this study will guide producers to select the best control options that can result in higher comparative growth and yield without compromising fruit quality. Further studies should be conducted to confirm the effects of the studied entomopathogens and plant extracts under varied agro-climatic conditions.

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Comparative postharvest responses of carnation and chrysanthemum to synthesized silver nanoparticles (AgNPs)

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Abstract: Carnation (*Dianthus caryophyllus* L.) and chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitam.] cut flowers are among the most important commodities that dominate flower markets throughout the world. Two major problems in the transportation and marketing of these flowers are their relatively short vase life and the rapid decline of their aesthetic value. In this respect, the current study investigates the effects of silver nanoparticles (AgNPs) on ethylene-sensitive (carnation) and ethylene-insensitive (chrysanthemum) cut flowers. Specifically, this research examines their morpho-physicochemical characteristics, antioxidant enzyme activities and vase life. Here, the AgNPs were synthesized by chemical methods and then applied on both flowers by a pulsing method. The treatments involved two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water), and the duration of exposure lasted for 24 h. Then, the flower stems were placed in an aqueous sucrose solution (4%) until the end of the experiment. All traits, except the vase life, were evaluated after 0, 3, 6 and 9 d following the treatments during the vase period. During this time, the control groups of both flowers showed considerable amounts of decrease in the relative fresh weight (RFW), vase solution uptake (VSU), flower diameter, membrane stability index (MSI) and total soluble carbohydrate (TSC). Meanwhile, there were increases in hydrogen peroxide content (H_2O_2) and peroxidase (POD) activity. The bacterial population of the stem end and total soluble protein (TSP) increased in carnation petals, but decreased in chrysanthemum petals. The activity of superoxide dismutase (SOD) dropped in carnation petals, whereas it rose in chrysanthemum petals. Using AgNPs at concentrations of 0.08 and 0.04 g L^{-1} can optimally extend the vase life of carnation and chrysanthemum, respectively.

1. Introduction

Nowadays, the floriculture industry is one of the most profitable sectors in horticulture. Its financial turnover in all respects amounts to over 300 billion dollars, and one-third of which is related to cut flowers (Chandler and Sanchez, 2012).Consumer demand for cut flowers and

ornamental plants are increasing, and the value of production is inevitably becoming higher. In this regard, flower quality is one of the most important indices in the sale and marketing of cut flowers. This makes flower quality an important factor in attracting customers over and over again (Scariot *et al*., 2014). Therefore, the advancement of post-harvest programs is a definite requirement within the cut flower market.

Even after their separation from mother plants, cut flowers continue to be metabolically active and proceed with all of their vital processes by consuming the available food in their tissues. Carbohydrates, proteins and fat can be used by cut flowers to meet their metabolic demands. The increase in vase life and the delay in flower senescence can be achieved by upholding the normal rate of water uptake, preventing the depletion of carbohydrate storage and limiting the exposure of flowers to ethylene (Halevy and Mayak, 1981).

Water balance is an essential factor in determining the quality and vase life of flowers. It should be maintained between water uptake and transpiration (Lu *et al*., 2010). Water uptake can be reduced due to the occlusion phenomenon generated by bacterial accumulation, physiological factors or air embolism (Damunupola and Joyce, 2008). Post-harvest senescence occurs within a few days, and is a major limitation in the marketing of cut carnation and chrysanthemum flowers. Stem end blockage is one of the important factors in early wilting of leaves and inflorescences in some cut flowers. van Doorn and Vaslier (2002) reported stem blockage is a major factor causing severe leaf wilting of chrysanthemum.

The in-rolling of petal margin and wilting of whole petals in senescence process in some cut flowers (e.g. carnation), is associated with the self-regulation of ethylene production (Yang and Hoffman, 1984), as a hormone, implying that the flowers are sensitive to ethylene. On the other hand, there are flowers such as chrysanthemum, pericallis and narcissus that initiate senescence without being influenced by ethylene, and thus are considered insensitive to ethylene (Dole and Wilkins, 2005; Jones 2013). Therefore, the short vase life and early wilting of inflorescences of carnation and chrysanthemum may be due to sensitivity of ethylene and stem end blockage, respectively. In fact, in ethylene-sensitive flowers, ethylene is a necessary requirement for the initiation and sustenance of senescence-based processes. The mechanisms by which ethylene can stimulate senescence involve changing the cell structure and increasing the concentration of reactive oxygen species (ROSs) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide (Abeles *et al*., 1992).

Studies have also shown that physiological changes occur during senescence. These include chlorophyll decomposition, decreased activity of antioxidant enzymes, increased productions of ethylene and ROSs, along with membrane damage to the cells of cut flowers (Prochazkova and Wilhelmova, 2007).

Advancements in nanotechnology, especially the development of silver nanoparticles (AgNPs), have led to a wide range of nanocomposites with antimicrobial properties. The high surface-to-volume ratio of these particles makes them biologically more active and increases their contact with fungi and bacteria (Chau *et al*., 2007). AgNPs are becoming popular in the flower industry for their ability to inhibit the synthesis of ethylene and delay the senescence in climacteric flowers (Kim *et al*., 2005). In addition, AgNPs tend to regulate the stomatal aperture, maintain the chlorophyll content, preserve the relative fresh weight (RFW) and membrane stability index (MSI), reduce the transpiration rate, weight loss, hydrogen peroxide(H_2O_2), Malondialdehyde (MDA) and ROSs, and increase the activity of antioxidant enzymes along with their effects on the vase life of different cut flowers such as gerbera (Liu *et al*., 2009; Solgi *et al*., 2009; Nazari and Koushesh Saba, 2017), rose (Lu *et al*., 2010; Nazemi Rafi and Ramezanian, 2013; Hassan *et al*., 2014), chrysanthemum (Carrillo-López *et al*., 2016), carnation (Naing *et al*., 2017; Lin *et al*., 2019 b), gladiolus (Li *et al*., 2017), peony (Zhao *et al*., 2018) and cut gardenia foliage (Lin *et al*., 2019 a). The application of AgNPs in vase solution of carnation alleviated vascular occlusion by inhibiting bacterial colonisation and biofilm formation on stem-end cut surfaces and in the xylem vessels (Lin *et al*., 2019 b). Maity *et al*. (2019) also showed that the *Piper betle* silver nanoparticles (PbSNPs) in vase solution of *Gladiolus* have played an important role for scavenging ROSs by enhancing antioxidant enzyme activities that led to decrease in MDA and increased the MSI.

Nanosponges are new nano-sized colloidal carriers synthesized from ß-cyclodextrins that have been prepared for delivering preservative and anti-ethylene compounds (Devecchi *et al*., 2009). Devecchi *et al*. (2009) evaluated the effect of nanosponges including anti-ethylene molecules, such as 1-methylcyclopropene (1-MCP), 1-methylcyclopentene (1-MCPt), 2,5-norbornadiene and $AgNO_3$ on vase life of carnation flowers. They concluded that, 1-MCP-

nanosponge complex outperformed the other treatments in extending the vase life. In addition, Seglie *et al*. (2011) reported that 1-MCP in cyclodextrin-based nanosponges improved the vase life of carnation cut flowers.

Nonetheless, no report has so far described comparisons between the responses of ethylene-insensitive and ethylene-sensitive flowers to AgNPs. Therefore, the current research is aimed an evaluating how the cut flowers of carnation (highly sensitive to ethylene) and chrysanthemum (insensitive or slightly sensitive) respond to the application of AgNPs by the pulsing method. Comparisons are made between the two types of cut flowers by measuring their morphological, physiological and biochemical properties, as well as their enzymatic activities.

2. Materials and Methods

Plant materials and application of treatments

The cut flowers of carnation (*Dianthus caryophyllus* cv. Yellow Viana) and chrysanthemum (*Dendranthema grandiflorum* cv. Boris Becker Sunny) were harvested at their commercial harvesting stage from a soilless-cultured greenhouse. They were taken to the laboratory and the basal ends of the stems were immediately cut to reduce the stem length to 40 cm. Apart from 3-4 leaves on the top of each stem, all other leaves were removed. Then, AgNPs were applied at two concentrations (0.04 and 0.08 g L^{-1}) by the pulsing method for 24 h. The flowering stems were individually put in a bottle vase containing 300 mL deionized water. This also contained 4% (w/v) sucrose until the end of the experiment. To prevent the evaporation and contamination of the vase solution, the vase opening was covered with aluminum foil. All traits, except for vase life, were evaluated after 0, 3, 6 and 9 d following the application of treatments. The pH of vase solution at the first day (d 0) was 7.82 and 8.76 for carnation and chrysanthemum, respectively. On d 9 of the vase period the pH of vase solution was 3.95 for carnation and 4.06 for chrysanthemum. The pulsing treatments and vase life were evaluated at 23±2°C, 50%±10% RH and 12 h of 15-20µmol $m^{-2}s^{-1}$irradiance from cool-white fluorescence lamps.

Synthesis of AgNPs

To a solution of 0.265 mM (0.045 g) of silver nitrate in 100 mL distilled water, 10 mL of a trisodium citrate aqueous solution (1%) was added slowly at room temperature. After 10 minutes, 0.2 mL of ascorbic acid (0.005 M) was added to the mixture of reaction and stirred for 1 h until a yellow-green AgNPs colloid was formed (Tavallali and Pouresmaeil, 2012). The surface morphology of AgNPs was indicated by scanning electron microscopy (SEM) (TSCAN, Czech Republic) and showed in figure 1, it is exhibited that AgNPs have a size of about 8-80 nm.

Measurement of flower and stem diameter

The flower diameter was measured in two directions, and the stem diameter was measured in three parts (i.e. under peduncle, middle, and end of the stem). These measurements were repeated every three days and the average of values were reported.

Measurement of relative fresh weight

The relative fresh weight (RFW) of flowering stems in both types of cut flower was calculated every three days by the following formula:

$$RFW\ (\%) = (Wt/Wt_0)\times 100$$

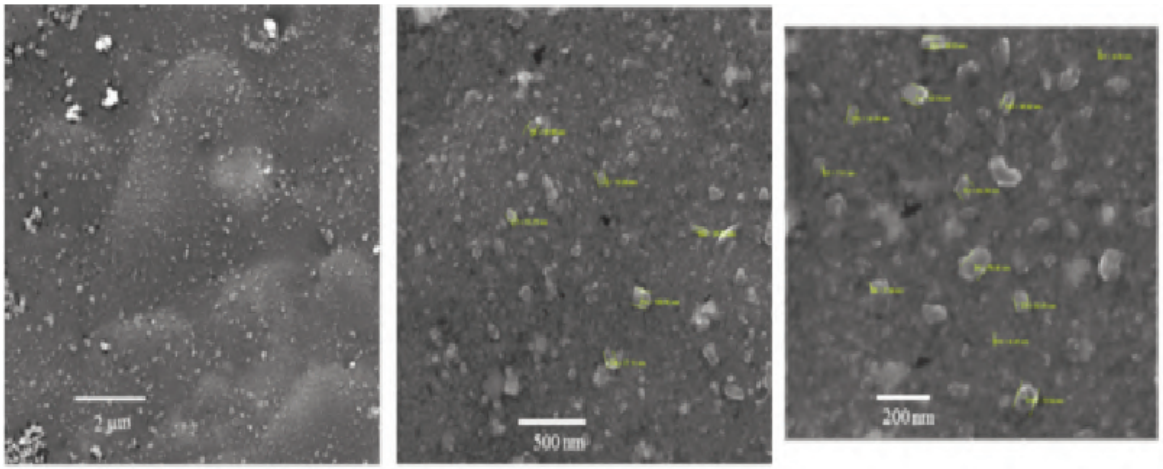

Fig. 1 - The SEM image of AgNPs.

where W_t is the weight of the stem (g) at t = d 0, 3, 6, and 9,while the $Wt_0$is the weight of the same stem (g) at t = d 0 (He *et al*., 2006).

Measurement of vase life

The vase life of both types of cut flower was measured by counting the number of days from the beginning of the experiment until 50% of the flower had wilted. In carnations, the occurrence of in-rolling and browning petals by more than one-third was considered as the end of the vase life (Naing *et al*., 2017). In chrysanthemum, however, the vase life ended when 50% of petals had wilted and the leaves had yellowed (Carrillo-López *et al*., 2016).

Measurement of vase solution uptake

The weight of the vase with and without the flower shoots was recorded daily, and the following formula was used for calculating the vase solution uptake (VSU): VSU (mg g^{-1} stem f. w.) = (S_{t-1}-S_t); where S_t is the weight of vase solution (g) at t = d 0, 3, 6 and 9, S_{t-1} is the weight of vase solution (g) on the previous day (He *et al*., 2006; Lu *et al*., 2010).

Measurement of membrane stability index

To measure the membrane stability index (MSI) of petals, this method involved the preparation of petal discs (measuring 1 cm in diameter) which were placed in falcons containing 20 mL distilled water. After this, a series of falcons were placed in a warm bath (40°C) for 30 min and their electrical conductivity (EC) was read by a conductivity meter (*C1*) after the falcons had cooled down to 25°C. Then, the second series of falcons was placed in a warm bath of 100°C for 20 min and their EC was read after having cooled down to 25°C (*C2*). In the end, the MSI was calculated using the following equation (Sairam *et al*., 2002):

$$MSI= [1-(C_1/C_2)] 100$$

C_1 = EC after exposure to 40°C and C_2= EC after exposure to 100°C.

Measurement of bacterial population of stem end

To measure the bacterial population on the stem end, one gram of the stem end was homogenized and diluted with peptone water until a concentration of $10^{-3}$was reached. Subsequently, 1 mL of this solution was transferred to Petri dishes and then a volume of 10 mL sterilized plate count agar medium was added to each Petri dish. These were slowly mixed for 5 to 10 s. The cultured Petri dishes were then kept in an incubator at 32°C for 2 d and, after counting the bacterial colonies, the results were reported as log CFU g^{-1} (Balestra *et al*., 2005; Liu *et al*., 2009).

Measurement of total soluble protein

According to the Bradford method (1976), 0.5 g of petal tissue was powdered with liquid nitrogen, and then 0.25 g of polyvinylpyrrolidone (PVP) was added to the solution when stirring the 1.5 mL of potassium phosphate buffer containing sodium metabisulfite (0.019 g per 100 mL buffer). The homogenized samples were centrifuged (HETTCH, Germany) at 4°C for 20 min at 15,000 g. Then, 50 µL of supernatant was mixed with 950 µL of Bradford solution and, after 15 min, the light absorption was read at 595 nm by a spectrophotometer (UNICO 2100, USA).

Measurement of peroxidase activity

To measure the peroxidase (POD) enzyme activity, according to a method reported by Hemeda and Klein (1990), first 400 µL of 50 mM potassium phosphate buffer (pH 7) was mixed with 40 µL of 1% glycol and 40 µL of 0.3% hydrogen peroxide in an ice bed. Then immediately, 65 µL of protein extract was added to the mentioned composition. The changes in light absorption were read at 120 nm by a spectrophotometer within a wavelength of 470 nm.

Measurement of superoxide dismutase activity

The activity of superoxide dismutase enzyme (SOD) was measured by the Beyer and Fridovich (1987) method. According to this method, the solution used for the reaction was prepared by mixing 25 mL of 50 mM phosphate buffer (pH 7) with 0.0035 g L-methionine (9.9 mM), 0.004 g NBT (57 µM) and 7.5 µL Triton X-100. One mL of the reaction mixture was blended with 10 µM riboflavin and 20 µL of protein extract. The mixture was placed on a shaker at a distance of 30 cm from a 20-watt fluorescent lamp for 10 min. Then, the light absorption was measured at 560 nm by a spectrophotometer.

Measurement of total soluble carbohydrate

To measure total soluble carbohydrates (TSC), 0.5 g of petal tissue was crushed using liquid nitrogen along with 5 mL of 95% ethanol which helped obtain uniform extracts. The supernatant extract was centrifuged for 10 min at 3,500 g. Then, 1 mL of this extract was combined with 3 mL of Anthrone before being transferred to a warm bath of 100°C. The heat caused the appearance of a colored phase after 10 min. Subsequently, the samples were removed from the warm bath and were allowed to cool down at room temperature. Their light absorption was read at 625 nm. The TSC content of petals was determined by creating a standard curve using standard glucose.

The results were expressed as mg g^{-1} f.w. (Irigoyen *et al*., 1992).

Measurement of hydrogen peroxide

In order to measure the amount of hydrogen peroxide (H_2O_2), the method was similar to the one used by Alexieva *et al*. (2001). Accordingly, 0.2 g of petal tissue was completely ground with 5 mL trichloroacetic acid (TCA). The extract was obtained and centrifuged at 10,000 g for 5 min. Then, 250 µL of the supernatant was mixed with 250 µL of 100 mM phosphate-potassium buffer (pH7) and 500 µL of 1 M of potassium iodide (KI). The absorbance of each sample was read by a spectrophotometer within a wavelength of 390 nm.

Statistical analysis

This research was conducted as a factorial based on a completely randomized design (CRD), it had three factors that the first factor was the type of flower at 2 levels (carnation and chrysanthemum), second factor was AgNPs at 3 levels (0.04 and 0.08 g L^{-1} along with deionized water) and the third factor was the sampling time at 4 levels (0, 3, 6 and 9 d of the vase period) with 3 replications, each of which included 8cut flower stems. For measuring the vase life, two factors of the type of flower and the sampling time were not considered and its design was as a CRD with three treatments (AgNPs at 0.04 and 0.08 g L^{-1} along with deionized water). Data were analyzed using SAS software and a comparison of mean values was made by the LSD test at 5% probability level.

3. Results

Relative fresh weight

Based on the comparison of mean values, the relative fresh weight (RFW) of both cut flowers gradually decreased in the control treatment during the vase period. A faster rate of this decline was observed in carnations, as compared to the chrysanthemum. From the initial days to the ninth day, the decrease of RFW in carnations was about twice as much as the decrease in chrysanthemum. The application of0.04 and 0.08 g L^{-1} AgNPs caused the percentage of RFW to remain relatively constant in both cut flowers until the third days, but then the RFW gradually decreased. Furthermore, treating the cut flowers with AgNPs caused a better preservation of their RFW from d 3 to d 9 of the vase period, as compared to the control, but the difference between the two concentrations was no significant. On d 9 of the vase period, the RFW of chrysanthemum was heavier than that of carnations, and the differences were significant (Fig. 2).

Vase solution uptake

There was an increase in the rate of vase solution uptake (VSU) by both flowers until the third day of the vase period, by which time the VSU in carnations was almost twice as much as that in chrysanthemum. From the third day onward, the VSU in both flowers decreased. The application of AgNPs caused an increase in the VSU in both flowers compared to the control. On d 9 of the vase period, the VSU in carnations had been significantly affected by both concentrations of AgNPs, whereas the chrysanthemum was only affected by 0.08 g L^{-1} AgNPs to a substantial degree (Fig. 3).

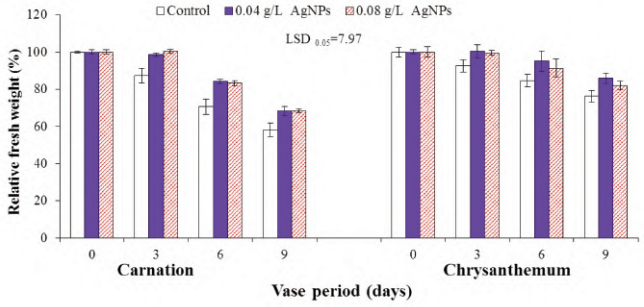

Fig. 2 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on RFW of two cut flowers during the vase period. Values represent means ± standard error (n=3). Least significant difference (LSD) at P≤0.05 was used for means comparison.

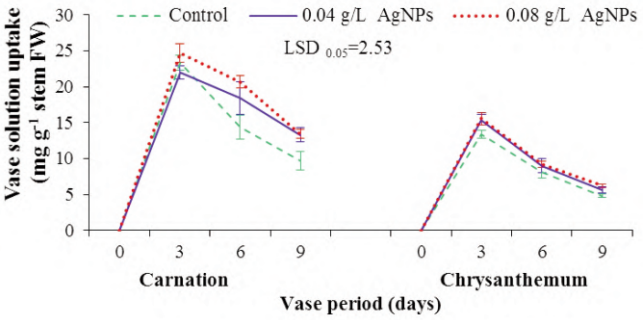

Fig. 3 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on VSU of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

Bacterial population of stem end

The bacterial population of the stem end in carnations increased throughout the vase period. In chrysanthemum, however, the same trend continued until the third days before declining. Both concentrations of AgNPs reduced the bacterial population of the stem end in chrysanthemum during the vase period, making it significantly different when compared to the control on d 9. On the other hand, this trait in carnations was only affected by 0.08 g L^{-1} AgNPs to make a significant difference compared to the control. In general, in both flowers, 0.08 g L^{-1} AgNPs was more effective than the 0.04 g L^{-1} in reducing the bacterial population of the stem end. The highest of this trait among both cut flowers was observed in the control group of chrysanthemum on d 3, whereas the lowest of this value was caused by 0.08 g L^{-1} AgNPs and measured on d 9 (Fig. 4).

Changes in the stem and flower diameter

The stem diameter was one of the traits which was not significantly affected by AgNPs in both flowers. Even a comparison between d 0 and 9 of the control groups showed no significant difference. Despite the fact that 0.08 g L^{-1} AgNPs in both flowers caused the stem diameter to become thicker in comparison with the stems of plants treated by 0.04 g L^{-1} and the control, there were no significant differences between these groups (data not shown).

From d 3 onward, the diameter of both flowers decreased. However, it occurred more dramatically in carnations which shrank twice as much as the chrysanthemum. The effect of AgNPs on changing the flower diameter was significant only in carnations, during their vase period. Although AgNPs increased the diameter of flowers in both species, as compared with their respective control groups, the diameters had finally decreased by d 9 of the vase period. On the mentioned day, these treatments had made a significant difference in carnations only, as compared to the carnations control group. In general, the biggest flower diameter was observed in chrysanthemum on d 0 after being treated with 0.04 g L^{-1} AgNPs, and the smallest of all diameters was obtained in the control group of carnations on d 9 (data not shown).

Membrane stability index

The decline in values of the membrane stability index (MSI) occurred in both flowers during the vase period, and no significant differences were observed between the two flowers in this respect. In the beginning of the vase period (d 0), the MSI in carnations was higher than in chrysanthemum, but the value of this trait decreased in both flowers through time. On d 9, this decrease was twice as much in carnation when compared to chrysanthemum. In both flowers, AgNPs caused the MSI to increase during the vase period, as compared to the control, but chrysanthemum responded more strongly to the treatment than the extent to which carnations did. In response to AgNPs, chrysanthemum showed a gradual increase in the MSI value - so much so that it became slightly $^{-1}$ AgNPs can be a successful treatment for increasing the MSI in carnations. In contrast, however, the concentration of 0.04 g L^{-1} worked optimally on chrysanthemum. In general, the highest value of MSI was observed in carnations on d 0 when treated with 0.08 g L^{-1} AgNPs, whereas the lowest value occurred in the control on d 9 (Fig. 5).

Total soluble protein

A comparison of the mean values showed that the

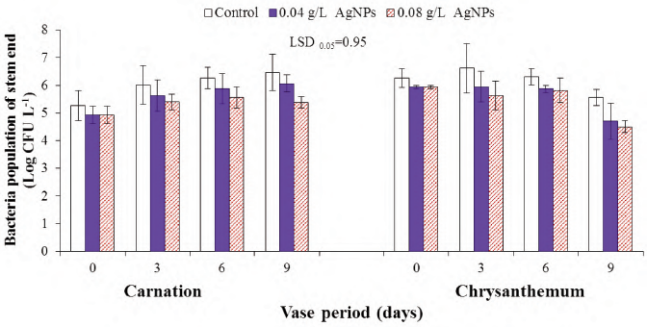

Fig. 4 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on bacterial population of the stem end of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

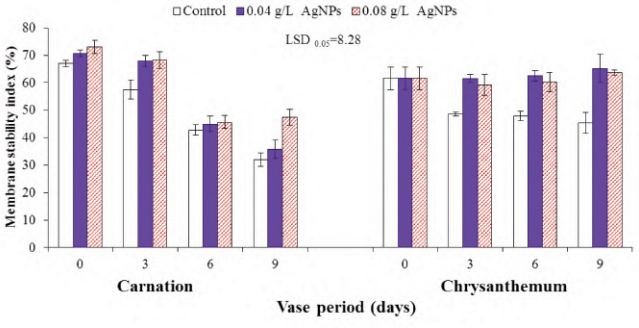

Fig. 5 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on MSI of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

content of total soluble protein (TSP) in chrysanthemum petals was about 4 times higher than that of carnation petals on d 0. In carnations, the TSP content of the petals increased during the vase period. Meanwhile, chrysanthemum underwent a different pattern of change, whereby the content of TSP increased from d 0 to d 3, but then decreased sharply until d 6, and continued to decline at a gradual rate until d 9. The AgNPs increased the TSP in both flowers during the vase period, as compared to the control. Generally, the highest value of TSP was observed in chrysanthemum on d 3 in the treatment group of 0.04 g L^{-1} AgNPs, whereas the lowest value was observed in the control on d 9 (Fig. 6).

Total soluble carbohydrate

The total soluble carbohydrate (TSC) content in carnation petals was more than the content in chrysanthemum petals. In the control groups of both flowers, the content of TSC decreased. The use of AgNPs in both flowers did not cause a significant difference in TSC on d 0, as compared with the control, but thereafter the difference gradually became significant until d9. Applying the AgNP at 0.08 g L^{-1} on carnations and at 0.04 g L^{-1} on chrysanthemum significantly increased the TSC in comparison with their respective control groups. In general, carnations responded more strongly to the use of AgNPs, and the increase in their TSC content was much greater than in the case of chrysanthemum. The highest TSC content was measured in carnations on d 3 of the vase period after being treated with 0.08 g L^{-1} AgNPs. The lowest content was measured in the control group of chrysanthemum on d 9 (Fig. 7).

Hydrogen peroxide content

The petals of both flowers initially contained similar amounts of hydrogen peroxide (H_2O_2) which gradually increased during the vase period. The rate of this increase was higher in carnations compared to the chrysanthemum. When comparing the H_2O_2 content between d 0 and d 9, its increase in carnations was about three times more than the increase measured in chrysanthemum. The application of AgNPs on both flowers reduced the H_2O_2 content in their petals, as compared with the control, but the increase was not prevented completely. On the last day of the vase period, the application of AgNPs on carnations led to a significant difference in H_2O_2 content when compared with the control. However, this was not the case in chrysanthemum. In general, the highest amount of H_2O_2 was measured in the control group of carnations on d 9 of the vase period. Its lowest amount was obtained in chrysanthemum on d 6 by 0.08 g L^{-1} AgNPs (Fig. 8).

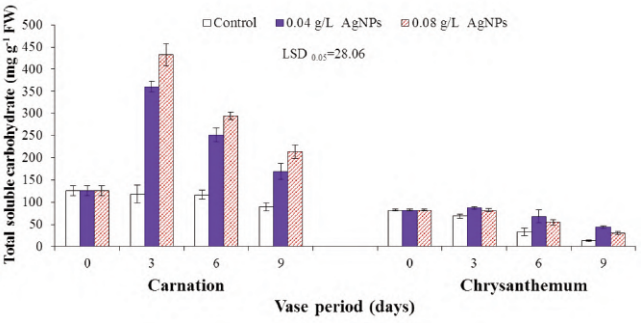

Fig. 7 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on TSC of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

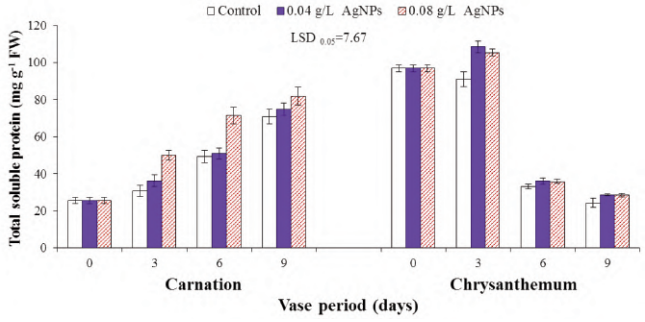

Fig. 6 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on TSP of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

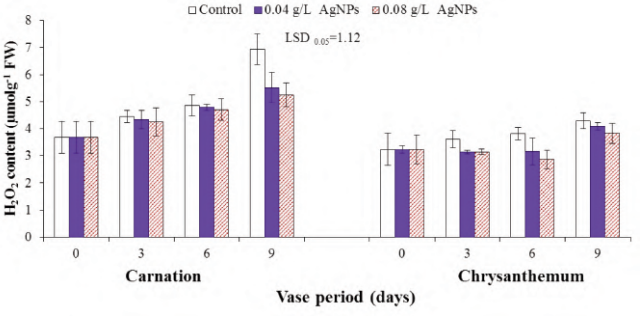

Fig. 8 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on H_2O_2 content of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

Peroxidase activity

Based on the results, peroxidase (POD) activity in both flowers increased until d 3 of the vase period and then decreased until d 6 before increasing again thereafter. Using AgNPs on chrysanthemum, unlike carnation, significantly increased the activity of POD in comparison with the control. When treated with 0.04 g L^{-1} AgNPs, the chrysanthemum showed a level of POD activity on d 9 that was about 2.5 times greater than the activity in carnations on the same day. In general, the highest level of POD activity was measured in chrysanthemum on d 9 after the treatment with 0.04 g L^{-1} AgNPs, whereas the lowest level of activity was measured on d 6 in the control (Fig. 9).

Superoxide dismutase activity

Based on the results, that the superoxide dismutase (SOD) activity in carnation petals was about 2.5 times higher than that of chrysanthemum petals on d 0, but on d 9 it was completely different, and the activity of this enzyme in chrysanthemum was more than 4.5 times that of carnation. Generally the activity of SOD dropped in carnation petals, whereas it rose in chrysanthemum petals. The AgNPs decreased the SOD in both flowers during the vase period, as compared to the control. Generally, the highest value of SOD was observed in chrysanthemum on d 9 in the control treatment, whereas the lowest value was observed in carnation on this day of vase period in the treatment group of 0.08 g L^{-1} AgNPs (Fig. 10).

Vase life

Clearly, AgNPs caused the vase life of both flowers to increase. Both concentrations had a significant effect on carnations, while chrysanthemum was significantly affected by the 0.04 g L^{-1} only. In carnations, the vase life increased parallel to the increase in applied concentrations of AgNPs from 0.04 to 0.08 g L^{-1}, the effects of which were significantly different compared to each other. Using 0.08 g L^{-1} AgNPs yielded more appropriate results in carnations. On the other hand, the vase life of chrysanthemum was slightly affected in a negative manner as the concentration of AgNPs rose from 0.04 to 0.08 g L^{-1}, but the difference between the two concentrations was insignificant. Nonetheless, both caused significant differences in comparison with the control (Fig. 11A and B).

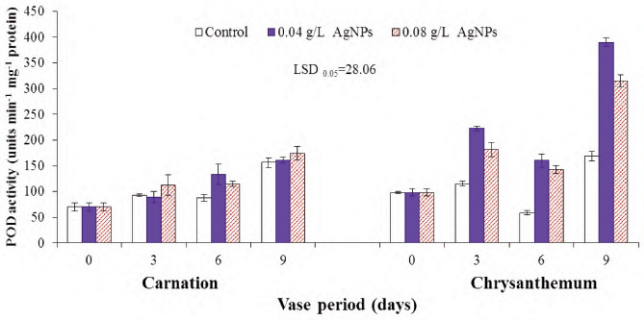

Fig. 9 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on POD activity of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

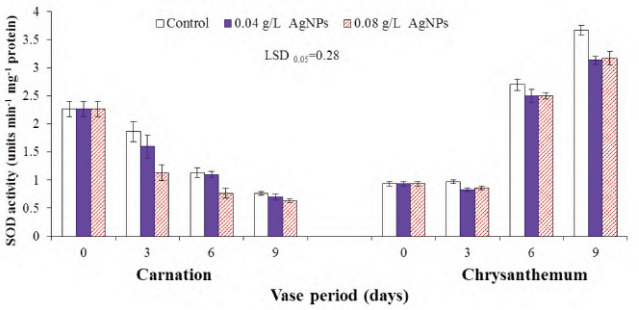

Fig. 10 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on SOD activity of two cut flowers during the vase period. Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

4. Discussion and Conclusions

In this study, the cut flowers of carnation and chrysanthemum showed various levels of decrease in RFW (Fig. 2), VSU (Fig. 3) and flower diameter throughout the vase period. Similar results have been reported after assessing the RFW and VSU of gerbera cut flowers (Liu *et al*., 2009), rose (Chamani *et al*., 2005) and cut gardenia foliage (Lin *et al*., 2019 a) during the vase period. The current study showed that using the AgNPs reduced the bacterial population of the stem end in both flowers, while the RFW was improved as compared to the control. Previous studies confirm such findings on the use of AgNPs in cut flowers such as *Gerbera* (Liu *et al*., 2009; Solgi *et al*., 2009; Nazari and Koushesh Saba, 2017) and rose (Lu *et al*., 2010; Nazemi Rafi and Ramezanian, 2013; Hassan *et al*., 2014). These studies showed that the vase life improves when the bacterial population of the stem end decreases, besides when the VSU and RFW increase.

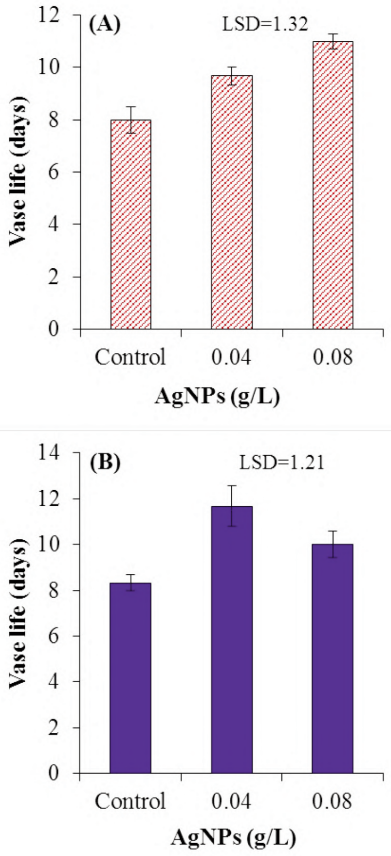

Fig. 11 - Effect of two concentrations of AgNPs (0.04 and 0.08 g L^{-1}) along with the control (deionized water) on vase life of carnation (A) and chrysanthemum (B). Values represent means ± standard error (n=3). LSD at P≤0.05 was used for means comparison.

The decrease in the RFW of cut flowers marks the beginning of senescence in flowers, and the more the flowers become closer to the process of senescence, their ability to take up water from the vase is reduced. The imbalance between water uptake and transpiration distorts cell turgor, thereby causing the flowers to wither (Reid and Jiang, 2012). The survival of cut flowers depends largely on a positive water balance: an increase in water uptake and a decrease in water loss (Halevy and Mayak, 1981; Van Doorn, 1997). After being placed in the vase, the flower loses RFW in part because of vascular occlusion and the growth of microorganisms which grow in the vase solution. They reduce the water uptake by blocking the stem end and vessels, thereby causing water stress which is a main factor in the reduction of vase life (van Doorn, 1997; Macnish *et al*., 2005). Adding a germicide to the vase solution may control the activity of microbes. It has been reported that adding antimicrobial compounds containing Ag^+ ions to the vase solution improves the vase life of cut flowers (Hassan *et al*., 2014). AgNPs nanoparticles function effectively because of their high surface-to-volume ratio and their crystallographic surface structure (Rai *et al*., 2009). Furthermore, Ag^+ ions cause bacterial cell death by affecting membrane structure and permeability, inhibiting DNA transcription, disrupting transport activity and changing cellular content and ATP (Feng *et al*., 2000; Sondi and Salopek-Sondi, 2004; Rai *et al*., 2009; Dakal *et al*., 2016).

This difference in the bacterial population of the stem end of the two cut flowers may depend on the genotype of the plant, as well as the extract and metabolites that are secreted from the stem end and are released into the vase solution, thereby affecting the growth of bacteria. Phenolic compounds are secondary metabolites that are involved in plant defense against pathogens (Naczk and Shahidi, 2004). In traditional Chinese medicine, the flowering heads of *Chrysanthemum indicum* are used as a source of bactericide, with additional antifungal and antiviral properties (Shunying *et al*., 2005). In agreement with our results, Shunying *et al*. (2005) showed that the chemical composition and secondary metabolites of *Ch. indicum* possess antimicrobial activity.

Flower diameters in this study were significantly influenced by AgNPs in carnation flowers, butthe effect on chrysanthemum was not significant. Dar *et al*. (2014) reported that the flower diameter in *Dianthus chinensis* first increased and then finally decreased during post-harvest development and senescence, respectively. The current research showed that the flower diameter of carnations tends to increase after being treated with AgNPs. This accords closely witha previous report on *Polianthes tuberosa* which was treated with 0.015 g L^{-1} AgNPs and led to a significant increase in the diameter of flowers (Bahrehmand *et al*., 2014).

It is well known that the MSI gradually decreases from the time when the flowers open to the time of their senescence. Such a trend occurs evidently in certain flowers such as *Lilium* (Bieleski and Reid, 1992), rose (Hassan *et al*., 2014) and Iris (Ahmad and Tahir, 2016). The senescence process is mainly associated with protein loss, increased lipid peroxidation, membrane leakage, cell wall component degradation and cellular membrane disruption (Buchanan-Wollaston, 1997). AgNPs may preserve the membrane stability by curbing the peroxidation of lipids (Hatami and Ghorbanpour, 2013). There was a lower percentage of MSI in carnation petals, compared to chrysanthemum, which may be due to the fact that carnations are highly sensitive to ethylene. The reason becomes clear when knowing that ethylene

degrades the cell membrane and increases its leakage (van Doorn and Woltering, 2008), thereby resulting in a lower value of MSI. However, AgNPs acts against the production of ethylene (Hassan *et al*., 2014). In fact, AgNPs induce an efficient cellular electron exchange mechanism which reduces electron leakage and, subsequently, limits the creation of ROSs. AgNPs also dys regulate lipid peroxidation and have a propensity to maintain the MSI (Lu *et al*., 2010; Hassan *et al*., 2014).

Previous studies have reported a decrease in TSP content and an increase in protease activity during the vase life of cut flowers (Wagstaff *et al*., 2005; Dar *et al*., 2014; Zhao *et al*., 2018). Quite differently, however, we concluded that changes in the TSP content may vary depending on the type of flower, species and cultivar. Still, contrary to our results, Dar *et al*. (2014) showed that protein degradation in *Dianthus chinensis* is a key factor in regulating the senescence process of flowers. Shahri *et al*. (2011) also reported that TSP decreased during the senescence process of *Helleborus orientalis* cv. 'Olympicus', while there was an increase in its low-molecular-weight proteins. Accordingly, realizing an increase of TSP content in carnation petals could be a result of these low-molecular-weight proteins.

It has been shown that in flowers such as *Helleborus orientalis* and *Dianthus chinensis*, the TSC is reduced during the senescence process (Shahri *et al*., 2011; Dar *et al*., 2014).There was a lower content of TSC in chrysanthemum petals, as compared with carnations, which may be due to the genetic differences between the two plants, as well as the difference in the rate of polysaccharide decomposition during the opening and development of flowers and petals. In agreement with our results, it has been found that the starch and fructan polysaccharides are degraded and reduced during the development of flowers and the expansion of petals in chrysanthemum (Trustyl and Miller, 1991). Therefore, in this study, AgNPs in both flowers may have benefited the vase life by maintaining the content of TSC in petals and by promoting the mechanisms through which carbohydrates stabilize the cell membrane (Ashraf *et al*., 2010). Furthermore, AgNPs regulate and protect the cellular osmotic potential, inhibit the formation of free radicals (Parida and Das, 2005) and regulate the expression of genes (Rahdari *et al*., 2012).

Usually, the amount of H_2O_2 increases in plant cells during senescence (Ezhilmathi *et al*., 2007; Saeed *et al*., 2014). It can be suggested that high levels of H_2O_2 in carnation petals occur because of the plants sensitivity to ethylene. This can be compared with chrysanthemum which is not sensitive to the hormone. Our results showed that the use of AgNPs on both flowers reduced the production of H_2O_2 in petals, as compared with the control. These results accord closely with a previous report by Hassan *et al*. (2014) where the production of H_2O_2 became significantly limited in roses because AgNPs was used.

Senescence is an oxidation process in which ROS and antioxidant systems are involved (Buchanan-Wollaston, 1997). Plant cells have developed a series of antioxidant mechanisms for defense to prevent the production of ROSs and to limit their destructive effects on proteins, fats and nucleic acids (Arora *et al*., 2002). The enzymatic part of this system consists of antioxidant enzymes such as SOD, POD, catalase (CAT) and ascorbate peroxidase (APX) which degrade all types of ROSs (Balakhnina and Borkowska, 2013). Depending on the species or type of cultivars, the POD activity and SOD enzymes can exhibit different patterns of change in cut flowers during the senescence process (Hassan *et al*., 2014). As the production of H_2O_2 increased in the petals of both flowers during the vase period, the POD enzyme likewise increased its activity to scavenge the higher amounts of H_2O_2. Furthermore, Hassan *et al*. (2014) reported similar results which indicate that petals of the rose cut flower cv. 'First Red' - which is sensitive to ethylene (Chamani *et al*., 2005) - showed declining levels of SOD activity during the vase period. However, contrary to our results on the incremental trend of POD in carnations and chrysanthemum during the vase period, Hassan *et al*. (2014) reported that the POD activity in the rose cultivar decreases. It has also been reported that SOD activity in ethylene-sensitive flowers such as carnation is relatively lower compared to ethylene-insensitive flowers such as chrysanthemum. This comprises a major factor in accelerating the senescence (Bartoli *et al*., 1995).

In addition, it may be suggested that in ethylene-insensitive flowers such as chrysanthemum, ROS cause the greatest amount of damage to the cell components, thereby leading to a shorter vase life. For this reason, the SOD activity has to increase so as to create a parallel level of scavenging. In certain flowers such as carnations, which are sensitive to ethylene, the level of SOD activity may not be pronounced as much. As previously mentioned, AgNPs cause the plants to acquire an efficient cellular electron exchange mechanism, whereby the electron leakage and ROS production are reduced (Hassan *et al*., 2014). Perhaps, the activity of SOD does not

increase in carnations even when AgNPs are applied.

AgNPs increased the vase life of chrysanthemum and carnation (Figs. 11A and B) by increasing the RFW (Fig. 2) and reducing the bacterial population of the stem end (Fig. 4). The scientific literature contains several reports that mention the efficiency of AgNPs in increasing the vase life of gerbera (Liu *et al*., 2009; Solgi *et al*., 2009; Nazari and Koushesh Saba, 2017), rose (Lu *et al*., 2010; Nazemi Rafi and Ramezanian, 2013; Hassan *et al*., 2014), chrysanthemum (Carrillo-López *et al*., 2016), carnation (Naing *et al*., 2017) and gladiolus (Li *et al*., 2017).

In conclusion, AgNPs improved the vase life of both cut flowers by contributing to the values of their RFW, VSU, flower diameter, MSI, TSC, TSP and POD activity and by limiting their bacterial population of the stem end and H_2O_2 levels, as compared to the control. It is highly probable that AgNPs are capable of reducing the expression of genes responsible for the production of ethylene, as well as limiting the rate of transpiration and the opening of stoma. Furthermore, AgNPs have roles in regulating the aperture of the stoma and in reducing MDA production, thereby prolonging the vase life of cut flowers. Ultimately, this research revealed that the use of AgNPs at 0.04 g $L^{-1}$and 0.08 g L^{-1} can extend the vase life of at least two cultivars belonging to chrysanthemum and carnation, respectively.

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AHS
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Simulating the impact of projected West African heatwaves and water stress on the physiology and yield of three tomato varieties

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Abstract: Food security is a major issue in West Africa. As a consequence of climate change, increases in temperature and shifts in precipitation will have major ramifications for which crops can be grown in the region. Here we conducted an experiment to evaluate the impacts of short-term projected heat and water stress on three tomato varieties (*Solanum lycopersicum*): Oregon Spring, Roma VF, and Tropic. The plants were initially cultivated in the glasshouse programmed at 28/20°C day/night cycle. The treatments investigated were: control (CT); heat stress (Ht); water stress (Ws) and Heat + together with water stress (HtWs). For heatwave treatments, a 35/23°C day/night gradually taken up in a cycle was imposed. The water stress conditions were by decreasing the soil water field capacity by 50%. Leaf gas exchange and plant production parameters were measured. Our result indicated that all varieties suffered from significant declines in yield as consequence of the stresses. The heatwave treatment proved more detrimental on the tomato fruit yield than the water stress, except when these two treatments occurred in sequential cycles. The results of this study suggest that heatwaves and water stress, projected to occur more frequently due to climate change, may adversely impact the growth and yield of these three tomato varieties. Also, there was an unexpected fruit yield performance comparison among varieties tested in this experiment.

1. Introduction

Food security is a global issue that is projected to exacerbate as the human population grows (Godfray *et al*., 2010). To meet the United Nations Millennium Development Goal of "eradicating extreme poverty and hunger", considerable emphasis have to be placed on the developing regions of the world (Pingali *et al*., 2006), such as West Africa. West Africa comprises 15 countries and is home to an estimated 350 million people

(30% of the African continent) (United Nations, 2018). The population of this region is increasing rapidly, and is expected to reach 490 million by 2030 (Hollinger and Staatz, 2015).

In some part of West Africa, more than sixty percent of the population are dependent upon rain-fed agriculture, which is characterised by low fertilizer use, poor seed quality, inadequate water management, and low soil fertility (Benin, 2016). Agriculture is also negatively influenced by extreme weather events, such as heatwaves and droughts (Asare-Kyei *et al*., 2017). Indeed, the United Nations Food and Agricultural Organization reported that the drought occurred from June to August of 2019 in the Sahel region of West Africa, resulted in 9.7 million people being exposed to severe food insecurity, leading to 2 million children being under acute malnutrition (FAO, 2019).

Climate change poses additional concerns for food security in West Africa, particularly as the frequency and intensity of extreme weather events are projected to increase (Sylla *et al*., 2016). Combined with climate change, the growing human population characterized by rain-fed agriculture in West Africa, suggest that the eradication of poverty and hunger from this region will be challenging. Thus, adaptation to future climate plays a crucial role in securing food production (Easterling *et al*., 2007) and will require efficient adaptation strategies from the respective governments. While, some of these strategies have low or no cost and are already used in the region (e.g. shifting planting dates or selecting more resilient crop varieties), other strategies, such as developing new varieties or increasing irrigation, will require greater investment (Rosenzweig and Parry, 1994).

Tomato: a key horticultural crop in West Africa

Tomato (*Solanum lycopersicum* L.) provides substantial economic and nutritional benefits for humanity (Klunklin and Savage, 2017). This crop is one of the world's most highly consumed fruits (Arah *et al*., 2015), with over 177 million tonnes of production across ~5 million hectares of harvested land globally (FAOSTAT, 2017). Within West Africa, tomato is among the top ten horticultural crops in terms of yield. In 2016, 3.7 million tonnes were produced across 0.7 million ha (FAOSTAT, 2017). This crop is typically grown throughout West Africa under rain-fed conditions, with the greatest yield being achieved in the Sudano-Sahelian zone of West Africa, located south of the Sahara Desert and north of the humid Guinea region (Perez *et al*., 2017).

However, tomato growth and fruit production are affected significantly by climate (Petrozza *et al*., 2014) and yield is generally reduced in areas with extreme weather events (Oladitan and Akinseye, 2014) such as heatwaves (Hatfield and Prueger, 2015), flooding (Ezin *et al*., 2010), and drought, as well as pests and diseases (Ximénez-Embún *et al*., 2016). The optimum growth rate for most tomato varieties require temperatures between 20 and 27°C (Nicola *et al*., 2008) and 400-600 mm of water throughout their growing period (Jaria, 2012). Temperatures above 32°C can affect vegetative growth and reproduction of tomatoes (Pressman *et al*., 2002; Abdelmageed *et al.,* 2003; Müller *et al*., 2016), causing decreases in leaf area and plant development during the flowering stage (Nduwimana and Wei, 2017) and the failure of tomato fruit set (Sato *et al*., 2000). Water stress may lead to poor tomato plant growth and productivity through inhibition of cell expansion and reduction of stomatal opening (Chaves *et al*., 2003). Water stress also decreases the rate of photosynthesis, especially through stomatal conductance, as well as abundance of flowers and fruiting quality (Murshed *et al*., 2013).

Due to the importance of tomato for the West African region and the potential threat of climate change on its production, in this work we assessed the effect of heatwave and water stress on three varieties of tomatoes. We hypothesised that: (1) heatwave and water stress (i.e. less than 50% of soil water field capacity) will have a negative influence on the tomato varieties, causing reductions in fruit yield even after recovery period exposure; (2) the effect of heatwaves followed by water stress will be more severe than either of these stresses alone, causing a significant reduction in fruit yield; and (3) seasonally adapted varieties of tomatoes will respond differently to heatwaves and water stress, with varieties from warmer regions having higher yield than a cool-region variety.

2. Materials and Methods

Plant material and environmental conditions

We selected three varieties of tomato, two of which are commercially grown in West Africa ('Roma' and 'Tropic'), while the third ('Oregon Spring') is typically grown in cooler regions of the world. 'Roma' variety has a strong, compact stem with determinate vines (Gelmesa *et al*., 2010). This variety has been noted to be particularly suited to climatic conditions

in Savannah regions of West Africa (Ojo *et al*., 2013). *Tropic* was bred in the 1960's in the USA as an indeterminate variety adapted to warm, humid climates (Strobel, 1970). In contrast, *Oregon Spring* was bred as a determinate tomato for cold tolerance (Baggett and Kean, 1986).

The experiment was conducted under controlled environmental conditions at the Plant Growth Facilities glasshouse (PGF) at Macquarie University, Sydney, Australia. Seeds obtained from a seed distribution company called Eden Seeds, Australia, were germinated in a growth chamber at 22°C in commercial punnets. Six weeks after sowing, seedlings of similar height were transplanted to ten litres pots filled with 10 kg of soil mix that contained sand, topsoil, and rocky grey clay. Thirty-six plants of each variety were potted. The plants were initially cultivated in the glasshouse programmed at 28/20°C day/night cycle (temperature ambient, or TA). We kept a minimum night temperature of 20°C. Then from 04:00 h, temperature was increased by 0.5°C every 30 minutes, then remained constant at 28°C until 17:00 h. Following this, temperature was decreased by 0.5°C every 30 minutes to reach the minimum night-time temperature of 20°C. This temperature cycle was selected as it represents the conditions under which tomatoes are commonly grown in West Africa. In addition, a 12-hour photoperiod of 600 $\mu molm^{-1}s^{-1}$ and CO_2 concentration of 400 ppm (parts per million) were maintained in the glasshouses throughout the experiment.

After estimating the irrigation water capacity of the pots, all pots (with the exception of individuals in the water stress treatments, see details below) were watered daily to a 100% soil water field capacity (FC) which equated to a water holding capacity of 35%. The percentage water holding capacity was determined as the gain in the weight of the soil at saturation point divided by the dried weight of the soil x 100:

$$\% \text{ water holding cap.} = \frac{\text{gain in weight of the soil at saturation point}}{\text{dried weight of the soil}} \times 100$$

All plants were given the same quantity of fertilizer fortnightly using Yates Nutricote Standard Grey® fertilizer containing NPK (16:4:4) liquid fertilizer at the rate of 1 g L^{-1}.

Experimental design

Our goal was to assess the ecophysiological responses of the three tomato varieties to heatwave and water stress. For heatwave treatments, a 35/23°C day/night cycle (temperature high, or TH) was imposed, where temperature increased 1°C per hour from the minimum night temperature of 23°C from 04:00 h (local time) to the maximum day temperature of 35°C then kept constant at this till 17:00 h. Before been decreased at the same rate until the minimum temperature was reached (23°C), were it stayed constant again till the 04:00 h, then the cycle continued. This temperature range was selected based on future heat projections for West Africa (Abiodun *et al*., 2013; Sylla *et al.*, 2016).

Presently, there is no consensus regarding future rainfall patterns for West Africa (Roudier *et al.*, 2011), however increases in the frequency of drought conditions have been projected for the western Sahel sub-region (west of ~0°E) (Monerie *et al*., 2013). Thus, in this study, we simulated water stress conditions by decreasing the soil water field capacity by 50%.

To avoid location specific effects, the position of plants within the glasshouse were randomly rearranged weekly. Once reaching the flowering stage (~six weeks after sowing), three treatments together with the control were initiated for a total duration of eight weeks. Nine individual plants of each variety were placed in each treatment.

Ct - Control. Plants were grown at an ambient temperature (TA) consisting of a 28/20°C day/night cycle. All individuals were watered daily to approximately 100% FC. This condition was maintained for these Ct plants for the whole duration of the experiment. The treatment conditions are explained below:

1. *Ws - Water stress treatment*. Plants were grown at TA. The soil water field capacity of each individual was measured daily using a soil moisture sensor (Campbell Scientific Australia Pty Ltd -Hydro sense11®). During the treatment, all individuals were watered daily to only 50% FC. This condition was maintained throughout the eight weeks of treatment application.

2. *Ht - Heat treatment*. Plants were exposed to a cycle of a 14-day heatwave (TH = 23/35°C night /day) followed by 14 days at TA, and were kept well-watered. This cycle of conditions was maintained throughout the eight weeks of treatment application.

3. *HtWs - Heat and water stress treat*ment. Plants were exposed to a 7-day heatwave, during which they were well watered then followed by seven days of water stress (i.e. 50% FC) at TA. Also, this cycle of conditions was maintained throughout the eight weeks of treatment application.

After this treatments application period, all the plants were returned to the Ct conditions (see details

above) and allowed to recover for five weeks then the experiment terminated. The above conditions were simulated to imitate what normally happen in nature during a heatwave occurrence.

Ecophysiological and production measurements

Ecophysiological responses were assessed by measuring gas exchange traits, which included: transpiration rate, intracellular CO_2 concentration (Ci), stomatal conductance (*gs*), and net assimilation rate (*E*). These traits are widely used to evaluate physiological responses of plants to heat-water stress conditions (Nankishore and Farrell, 2016; Duan *et al*., 2017). The traits were measured on three mature, fully expanded leaves without damage and in good health, from five plants per treatment and variety using a Licor 6800 portable photosynthesis system (Li COR, Lincoln Nebraska, USA). Gas exchange measurements were taken at the third week (i.e. first week after complete treatments cycle-W3) and eight weeks (i.e. last week of treatment application-W8) between 09:30-14:00 h (local time). Also at the same period the number of flowers per plant were counted from the nine sampling plants per treatment and variety. Subsequently, the number of fruits that developed on these plants was also recorded. Maturity of fruit was determined based on a standard USDA colour chart (e.g. 'light red', UCANR, 2011). During the termination of the experiment (i.e. after the five weeks of recovery period), matured fruits were collected and weighed. The fresh and dry above ground biomass of the sampled plants were weighed and recorded too, with drying undertaken in an oven at 70°C for seven days. Measurements were noted as Fresh Biomass Weight Without Fruit (FWWF), Fresh Fruit Weight (FFW), Total Fresh Biomass Weight (TFW).

Statistical analysis

A two-way Analysis of variance (ANOVA) model with a correction formula called Satterthwaite approximation was performed to determine the effects of all the treatments (i.e. water treatment vs heat treatment vs both in sequential combination) vs control on the tomato varieties, using the *lmerTest* Package (Kuznetsova *et al*., 2017) in R version 3.4.3 (R Development Core Team, 2017). As measurements were taken on the same individuals over two different weeks, we used week as a covariate, with plant ID treated as a random effect. Post-hoc tests (Turkey contrasts) were used to compare means between treatments, and results were considered significant when $p < 0.05$. Statistical differences were reported as different letters on each figure. All figures were drawn in R using the 'ggplot2' (Wickham, 2016) packages.

3. Results

Ecophysiological measurements

From figure 1 and Table 1 it can be observed that for all the three varieties, transpiration rate differed significantly across treatments and weeks. Transpiration was generally highest among Ht plants and lowest in the HtWs treatment. For 'Oregon Spring', transpiration rates among Ht plants did not differ significantly to Ct, whereas rates were significantly higher among 'Roma's Ht plants. For both *Roma* and *Tropic*, transpiration rates between Ct plants and Ws were not significantly different (Fig. 1).

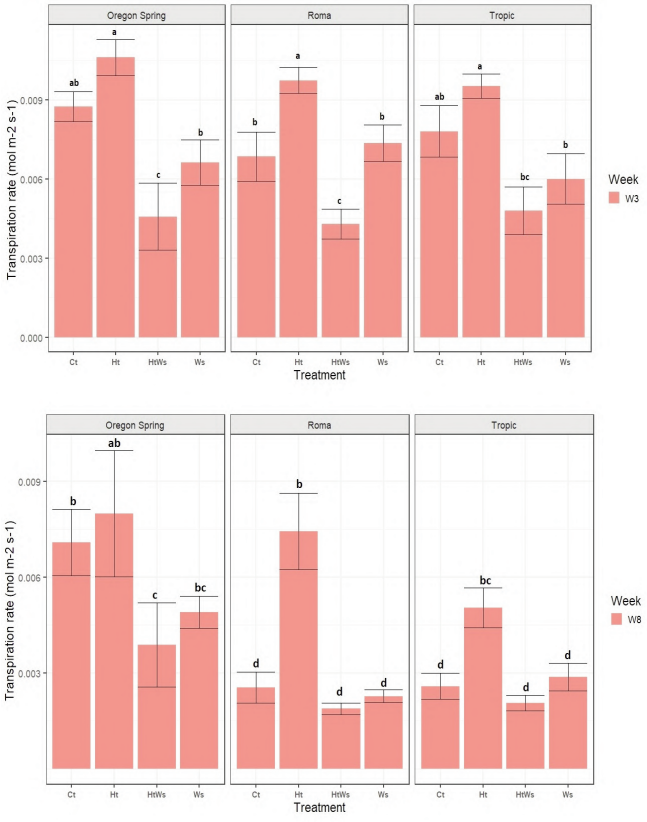

Fig. 1 - Transpiration rate (mol m^{-2} s^{-1}) of three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

Table 1 - Intracellular CO_2 response (Pa) of the three tomato varieties (Oregon Spring, Roma and Tropic)

Tomato varieties	Treatments		Weeks		Interaction	
	F-value	P value	F-value	P value	F-value	P value
Number of fruits						
Oregon Spring	399.681	< 0.001	350.074	<0.001	0.0466	<0.001
Roma	206.190	< 0.001	243.735	< 0.001	61.612	0.019
Tropic	289.064	< 0.001	453.048	<0.001	84.548	< 0.001
Number of flowers						
Oregon Spring	27.092	0.07	19.494	0.177	10.069	0.409
Roma	19.226	0.1568	27.351	0.1130	36.571	0.0289
Tropic	76.114	0.001	74.866	0.001	62.638	0.0033
Intracellular CO_2 response						
Oregon Spring	15.867	0.231	766.736	<0.001	12.037	0.3126
Roma	46.786	0.015	1.007.011	< 0.001	41.736	0.007
Tropic	41.423	0.023	11.552	0.285	0.9491	0.420
Assimilation Rates						
Oregon Spring	25.944	0.088	295.82	<0.001	7.449	<0.001
Roma	0.2935	0.829	911.11	<0.001	27.802	<0.001
Tropic	21.725	0.131	1322.9	<0.001	25.296	<0.001
Stomatal conductance						
Oregon Spring	59.115	0.006	119.84	<0.001	72.924	<0.001
Roma	81.714	0.002	131.58	< 0.001	7.443	<0.001
Tropic	37.627	0.032	259.27	<0.001	11.078	< 0.001
Transpiration Rates						
Oregon Spring	65.636	0.04	253.232	<0.001	13.997	0.247
Roma	23.232	<0.001	237.1	<0.001	9.214	<0.001
Tropic	13.053	<0.001	241.49	<0.001	5.393	0.002

The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

In general, plants exposed to heatwave stress had higher rates of intracellular CO_2 compared to plants in the other treatments. The lowest values occurred among HtWs plants, with similar patterns for the three varieties (Fig. 2). Stomatal conductance (g_s) across the three varieties was generally higher in Ht plants, and lowest in the HtWs treatment (Fig. 3, Table 1). However, treatment, week, and their interactions differed significantly for each variety. Among *Oregon Spring* and *Roma* varieties, Ct and Ht plants had significantly higher g_s than the HtWs plants. For *Tropic*, the Ht plants had significantly higher *g*s than HtWs plants in the weeks observed (Fig. 3, Table 1). Net assimilation rate (E) differed significantly for the weeks and the interaction between treatments and weeks among all the three varieties (Fig. 4; Table 1).

Production measurements

For the three varieties, the number of flowers was greater in both of the heatwave treatments (Ht and HtWs) compared to the Ct and Ws treatments. Ht treated plants had significantly the highest number of flowers. Also, by the end of the treatment application (w8), 'Oregon Spring' had an average of 8.5 (± 0.6 SD) flowers among plants in the Ws treatment,

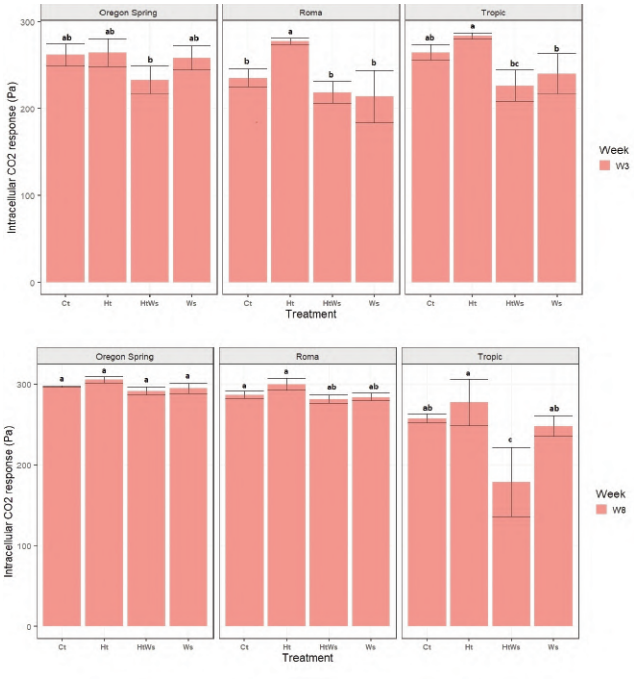

Fig. 2 - Intracellular CO_2 response (Pa) of the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

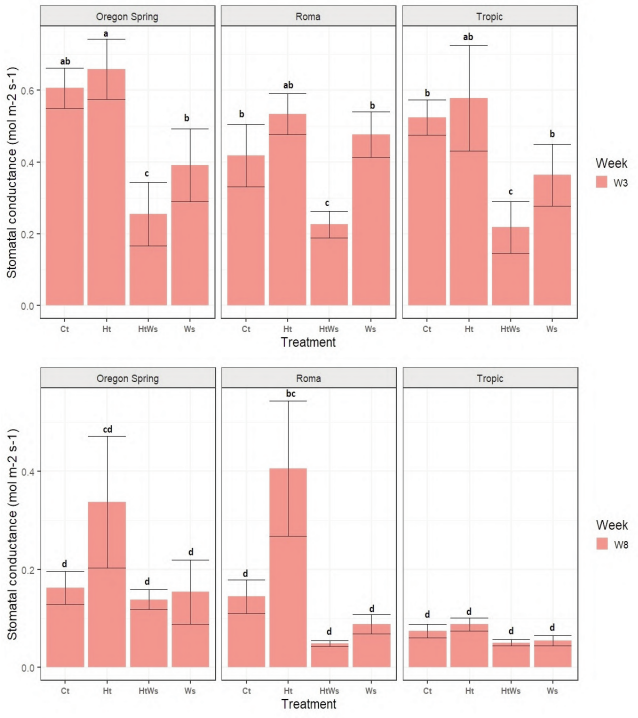

Fig. 3 - Stomatal conductance (mol m^{-2} s^{-1}) of the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

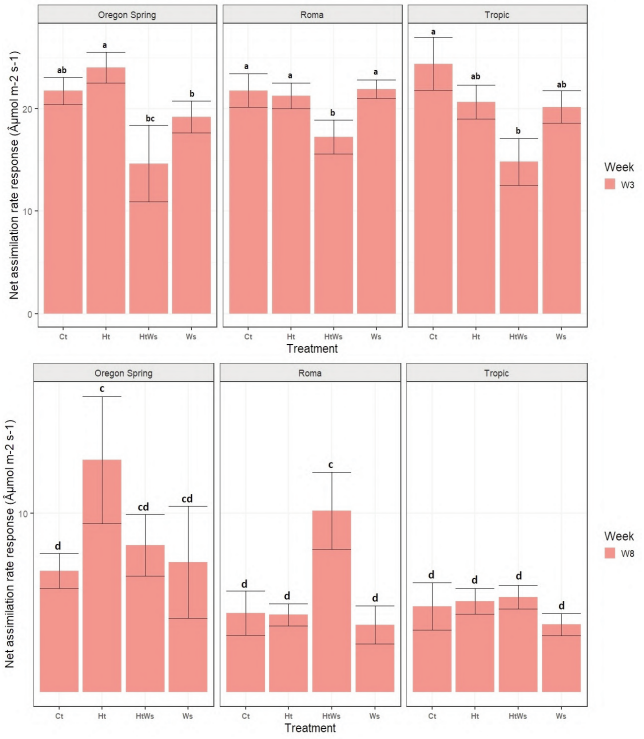

Fig. 4 - Net assimilation rate response (µmol m^{-2} s^{-1}) of the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

and 15 (± 4) and 20 (± 7) flowers on plants in the HtWs and Ht treatments, respectively (Fig. 5). For both 'Roma' and 'Tropic', the average number of fruits that developed per plant differed significantly across treatments, weeks, and their interactions (Table 1). All three varieties produced fewer fruits under both heatwave treatments. This effect was most pronounced for Roma where, by W8 of the treatment period, Ct plants had produced an average of 19.4 (± 8.9) fruits per plant compared to Ht plants (Ht: 2.6±2.4 and HtWs: 0.8±1.8), and significantly more fruits than plants in the Ws treatment (9.8±4.4). Oregon Spring produced more fruits in the heatwave treatments compared to the warmer varieties (Fig. 6, Table 1).

In general, heatwave treatments resulted in greater reductions to the harvested fruit weight than the water stress treatment. However, harvested fresh fruit weight (FFW) was higher among Ct plants compared to the other treatments (Fig. 7). This difference was significant for 'Roma' and 'Tropic' (Table 2), although there was no significant difference between Ct and Ws treatments for 'Oregon Spring'. For 'Roma' and 'Tropic' we found no significant differences among treatments when we compared Fresh Biomass weight without fruits (i.e. FWWF = Fresh Biomass excluding fruit). However, 'Oregon Spring' biomass was significantly greater for Ht plants than the other three treatments. For this variety, heatwave stressed plants were able to maintain growth at the expense of fruit production. We found a significant difference in the total biomass weight (TFW) (due to the adding of fruits weight) among treatments (Fig. 7, Table 2).

4. Discussion and Conclusions

We simulated the impacts of climate change by assessing the response of three varieties of tomato ('Roma', 'Tropic', 'Oregon Spring') to heatwave and water stresses. We found that assimilation rate, transpiration rate, intracellular CO_2 response, and stomatal conductance were all elevated under heatwave stress. In addition, we extended upon previous studies on tomatoes (e.g. Nankishore and Farrell, 2016;

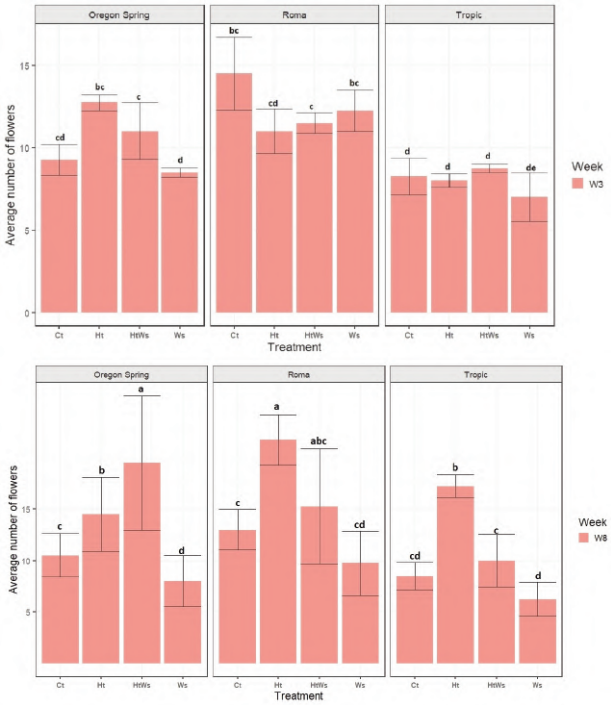

Fig. 5 - Average number of flowers per plant for the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

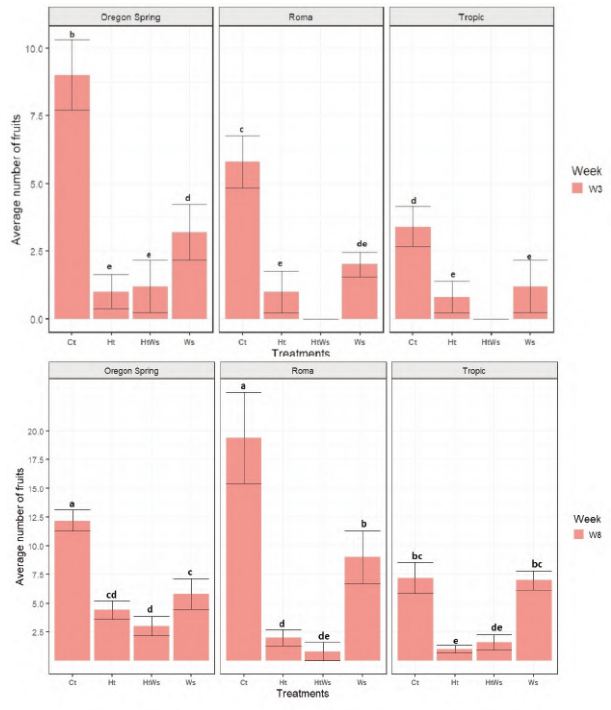

Fig. 6 - Average number of fruits per plant for the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). W3 and W8 = weeks of treatments imposition with measurements. Bars with the same letter are not significantly different at P<0.05 according to Turkey's test.

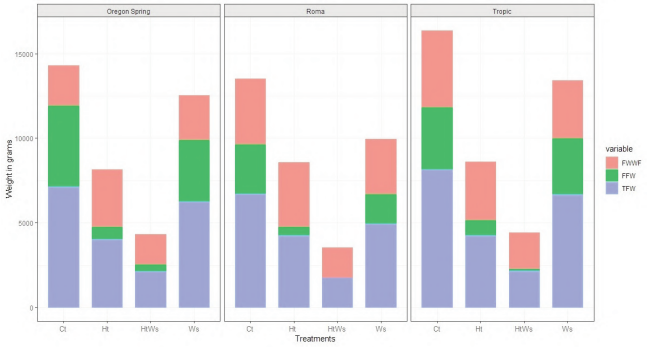

Fig. 7 - Fruit yield (marketable harvested fresh fruit) and aerial biomass accumulation response for the three tomato varieties (Oregon Spring, Roma and Tropic). The treatments consist of Control (Ct), Water stress (Ws), Heat + Water stress (HtWs) and Heat (Ht). Measured during the termination of experiment (i.e. after allowing for five weeks' recovery from the treatments). FWWF=Fresh Biomass weight without fruits; FFW=Fresh fruits weights; TFW= Total fresh biomass weight.

Table 2 - ANOVA (F-value and P-value) comparing the impact of the treatments on the harvested fresh fruits and aerial biomass measurements that developed on three varieties of tomato (OregonSpring, Roma and Tropic)

Tomato varieties	Treatments	
	F-value	P- value
Fresh weight without fruits (FWWF)		
Oregon Spring	3.04	0.046
Roma	1.18	0.335
Tropic	1.93	0.148
Harvested fresh fruits weights (FFW)		
Oregon Spring	14.66	<0.001
Roma	18.00	<0.001
Tropic	9.79	<0.001
Total fresh weight (TFW)		
Oregon Spring	6.75	0.002
Roma	19.89	<0.001
Tropic	10.96	<0.001

Sivakumar and Srividhya, 2016; Duan *et al*., 2017; Zhou *et al*., 2017) by assessing the impact of these stresses individually and sequentially on fruit yield, which is crucial from a socio-economic and food security perspective especially in region like West Africa. We found that these studied three tomato varieties experienced greater decline in yield (i.e. harvested fresh fruit weight) due to heat wave stress compared to water stress. However, under water stress fewer flowers were formed, indicating that heatwave stress results in a higher rate of aborted flowers compared with water stress, since its higher

number flowers did not transform to more fruits. Unsurprisingly, a greater impact on the plants fruit yield occurred when exposed to both stressors sequentially (i.e. heatwave then water stress together). Plants exposed to this treatment (i.e. HtWs) were unable to form fruit despite having more flowers than plants exposed to water stress only.

Water and heat stress affect photosynthesis and other physiological processes of tomatoes (Nankishore and Farrell, 2016; *Duan et al.*, 2017; Zhou *et al.*, 2017). Thus, as climate change intensifies, tomato yield in West Africa may decline due to the predicted higher frequency of heatwaves (Engelbrecht *et al*., 2015). High temperature can deactivate enzyme activity involved in the photosynthetic process, reducing or inhibiting photosynthesis (Rennenberg *et al*., 2006). This, in turn, has been reported to cause a 2.5% to 10% decline in yield for numerous crop species (Hatfield *et al*., 2011). In tomatoes, heat stress also has the potential to affect the viability of pollen (Hatfield and Prueger, 2015) resulting in failure of fruit set (Sato *et al*., 2000). Nevertheless, the impacts of heatwaves can be alleviated with irrigation. Here, we found that stomata closed when the plants in ambient temperature were under water stress, which invariably led to lower rates of leaf gas exchange. This was not the case for the plants under the heatwave treatment that remained well-irrigated, these plants consistently had higher values of stomatal conductance.

Crops differ in their capacity to recover from heatwave and water stress. For instance, some grains like maize (*Zea mays* L.) and rice (*Oryza spp*.) require a 10-day period of normal conditions (i.e. lower temperature and irrigation) after heat stress to enable the development of fruits (Hatfield and Prueger, 2015). For our experiment, a period of 14 days of normal conditions (i.e. 28/20°C day/night cycle and well-watered) between heatwave-imposed stress was not enough for plants to recover and produce fruits from the flowers formed, although the magnitude of the impacts on fruit production varied among the varieties. 'Tropic' and 'Oregon Spring' varieties had significantly higher fruit yield than 'Roma'. This finding highlights the vulnerability of these tomato varieties to heatwave and water stress. High temperature can affect allocation of resources, such as above- and below-ground tissues, with a tendency towards higher shoot-to-root ratios (Way and Oren, 2010). Neither heatwave or water stress, individually or sequentially, significantly affected vegetative biomass of the three tomato varieties, which contrasts to yield. Under all treatments within this experiment, the three varieties continued to have normal vegetative growth; however, flowers and the consequent fruit yield were drastically affected indicating the importance of optimal conditions (i.e. temperature and water) to facilitate plant reproduction (Peet and Welles, 2005; Parvej *et al*., 2010).

Initially, we hypothesised that heat stress would have a greater negative impact on the yield of 'Oregon Spring', the cool region variety, compared to the warm region varieties, i.e. 'Roma' or 'Tropic'. However, our results indicate the opposite, suggesting that *Oregon Spring* may out-perform either 'Roma' and 'Tropic' under similar conditions. This finding may indicate a higher plasticity in 'Oregon Spring' and a higher adaptive capacity. We suggest future research to explore the genetic characteristics of 'Oregon Spring' in response to heatwave and water stress to validate this as a potential variety to use under stress climatic conditions in as used in this experiment.

This study indicated that under a simulated climate projection of 50% less soil water field capacity and a heatwave of 35/23°C-day night cycles for West Africa, leaf gas exchange and fruit yield of three varieties of tomatoes were negatively affected, although vegetative growth was unaffected. Individually, heatwave stress was more detrimental for fruit yield than water stress, although experiencing these two stresses in sequence had an even greater consequence. Hence, ensuring plants are well watered can ameliorate some of the negative impacts of heatwave stress. Further studies are necessary to confirm the relationship existing among the various combinations of the heatwave and water stress conditions on different reproductive stages, such as pollen formation, pollen development and fertilization of tomatoes. Interestingly, the cool adapted variety assessed in this work, 'Oregon Spring', might represent an alternative option in warm temperature regions where 'Roma' and 'Tropic' varieties are underperforming, provided that irrigation is not limited.

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Different environments and doses of controlled-release fertilizer in peach rootstocks production

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Key words: fertilization, plant nutrition, *Prunus persica*.

Abstract: The objective of this study was to evaluate the effects of different environments and doses of controlled-release fertilizer (CRF) on the initial growth of peach rootstocks [*Prunus persica* L. (Batsch)] cv. Capdeboscq. The experimental design was completely randomized, in a 2 x 4 factorial design, four replications and five plants per plot. The treatments were the combination of two cultivation environments (on open-air benches and greenhouse benches) and four doses of CRF (0, 2, 4 and 8 g L^{-1} of substrate), in the 19-06-10 NPK formulation. Ninety days after their transplanting, the variables plant height, stem diameter, number of leaves per plant, shoot dry matter, root dry matter, total dry matter, plant height and stem diameter ratio were evaluated in addition to the Dickson Quality Index. All morphological variables evaluated presented a quadratic positive response to the increase of the applied fertilizer until the dosage of maximum technical efficiency (around 6.2 g L^{-1}). The maintenance of the plants in greenhouse benches and the incorporation of 4 g L^{-1} CRF to the substrate ensures greater efficiency in the input use, reducing the amount of time necessary for peach trees cv. Capdeboscq to achieve their grafting point and to be used as rootstocks.

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1. Introduction

Southern Brazil is the greatest national peach producer and this region is recognized as one of the main production centers of stone fruit trees in the country, especially peach trees. However, the traditional production system of these species is mostly performed in the field (Bianchi *et al.,* 2014; IBGE, 2018), strongly influenced by climatic conditions.

The use of protected environments is an alternative for the production of stone fruit trees, which can ensure the survival of the plants during the most critical phase of the tree production chain. Especially in rootstock production by seeds, the seedlings emergence stage and initial growth require environment control, as well as optimal nutrition and irrigation, and protection against pests and diseases in order to assure the fast growth and development of the plants (Souza *et al.,* 2017).

The production of fruit plants in protected environments, such as

greenhouses, allows the maintenance of optimum conditions for cultivation throughout the year, anticipating and extending the grafting period, reducing production costs and increasing the plants quality standard for sale (Oliveira *et al.,* 2017).

In Brazil, the traditional production system of stone fruit trees requires 360-540 days to grow a plant that is suitable for trading (Mayer *et al.,* 2015). In this system, the necessary time for rootstocks to reach the grafting point is of approximately 240 days (Fischer *et al.,* 2016). In part, this long period is due to the slow growth of plants in the field during the winter and early spring.

In order to reduce the time between seed germination and the production of rootstocks suitable for grafting, the cultivation in a protected environment, as well as the use of appropriate substrates and fertilizers in the proper doses for the crop are alternatives to optimize the initial phase of the plants growth (Bianchi *et al.,* 2014; Jamal *et al.,* 2017; Menegatti *et al*., 2019 b).

The environmental conditions of the plant production system directly influence the plants physiological processes and can directly affect plants growth (Souza *et al.,* 2017). Protected environments can promote greater uniformity for the plants growth in comparison with plants produced in the field or in an unprotected environment (Reis *et al.,* 2010; Fischer *et al.,* 2016; Oliveira *et al.,* 2017). Different environments can also affect the germination of seeds, and the growth and quality of the seedlings produced. The interaction between environmental conditions with the application of fertilizers may contribute to optimize the space for plant production in nursery and to reduce the plant production systems impact on the environment.

In addition to the use of commercial substrates, the increase of the nutrient supply is recommended because the substrate alone does not provide enough nutrients for the complete development of the plants (Dutra *et al.,* 2016). Among the many types of fertilizers available, the controlled-release fertilizer (CRF) is the most efficient. CRF promotes the slow release of nutrients and the absorption of the ideal amount throughout the plants' growth period, allowing them to achieve maximum strength (Zamunér Filho *et al.,* 2012; Menegatti *et al.,* 2017 a).

The concomitant use of fertilizers with the substrate favors the formation of more vigorous plants in shorter time, which reduces the period in which they stay in the nursery and, consequently, the production costs (Muniz *et al.,* 2013; Menegatti *et al.,* 2017 b). However, few are the researches that report the use of fertilization as an additional factor to the production of stone fruit trees (Zhang *et al.,* 2014; Jamal *et al.,* 2017; Menegatti and Bianchi, 2019). Even scarcer are the studies that consider plant propagation in a protected environment, such as a greenhouse in comparison with open environments (Picolotto *et al.,* 2007; Reis *et al.,* 2010).

The scarce information about the use of controlled-release fertilizers in the production of peach rootstocks in protected environments encouraged the accomplishment of this study, whose objective was to verify the effect of different environments and doses of CRF on the initial growth of peach trees cv. Capdeboscq for rootstock purposes.

2. Materials and Methods

Ripe peach fruits of cv. Capdeboscq were harvested in January 2017 from clonal mother plants kept in the Germplasm Collection of peach rootstocks at the Federal University of Pelotas (UFPel), Brazil. The experiment was conducted between October (2017) and January (2018), at the Department of Botany-UFPel, Capão do Leão, RS, Brazil, at 21° 48' south latitude, 41° 20' west longitude and an altitude of 11 m.

After the harvest of the fruits, the post-harvest management of the pits was carried out according to Picolotto *et al.* (2007). Then the seeds were stratified, as described by Souza *et al.* (2017). After the stratification period (35 days at 7°C), the seeds were sown, 1.0 cm deep, in 72-cell polystyrene trays (114 cm^3 per cell) containing a mixture of orchard soil + vermiculite + medium sand + commercial substrate Plantmax® (1:1:1:1) as substrate, and kept in a greenhouse.

When the seedlings, hereinafter referred as "plants", reached the transplant point (15 cm between collar and apex), they were transplanted into 1-liter plastic bags containing washed sand, which was used as substrate (Table 1) and whose CRF (Osmocote®) doses had the N-P-K formulation of 19-06-10 (4-6 months), which were previously incorporated into the sand.

The experimental design was completely randomized, in a 2 x 4 factorial design, with two environments (on open air benches and on benches inside the greenhouse) and four doses of Osmocote® (0, 2, 4 and 8 g L^{-1} substrate), with four replications and five plants per replication.

"Protected environment" refers to the Arco

Table 1 - Average chemical composition of the sand substrates used in the production of peach tree rootstocks

Substrates	OM** %	V %	H+Al mg dm^{-3}	SB mg dm^{-3}	CEC mg dm^{-3}	P mg dm^{-3}	K µg dm^{-3}	Ca µg dm^{-3}	Mg µg dm^{-3}	Zn µg dm^{-3}	Fe µg dm^{-3}	Mn µg dm^{-3}
Sand*	0.00	67.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.00	0.00

*sand; **OM= Organic Matter; V= Base saturation; SB= Sum of Bases; CEC= Cation Exchange Capacity (CEC).

Pampeana metallic structure greenhouse model, covered with a 150-millimeter thick, low-density polyethylene plastic film, arranged at the north-south direction and with the following dimensions: 10.0 m x 21.0 m and with the maximum height of 5.0 m. The benches used in the two environments were 1-meter high metallic structures, positioned at ground level. The environmental open air conditions and the ones in the greenhouse during the period of the experiment are described in Table 2.

Table 2 - Environmental conditions: temperature (T°C), relative humidity (RH%) and global radiation (W m-²) in the two cultivation environments (in open-air benches and greenhouse benches) during the cultivation period of peach trees cv. Capdeboscq

Environment	Temperature (°C)	RH (%)	Global radiation (W m-²)
Greenhouse	23.42	66.81	348.50
Open air	21.15	78.62	477.17

Ninety days after transplantation, when 75% of the plants of one of the treatments reached the grafting point (at least 5 mm of stem diameter and 10 cm above the soil), the plants were evaluated for the variables stem diameter (SD), plant height (H) and number of leaves (NL). Based on these data, it was possible to calculate the plant height and stem diameter (H/SD) ratio. The height of the rootstocks was measured using a graduated ruler, and the stem diameter was measured with a digital caliper.

The plants were dried in a forced air circulation oven at 70°C for 72 hours to obtain the shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) per plant. The Dickson quality index (DQI) was obtained by the formula: DQI = TDM/[(H/SD) + (SDM/RDM)], according to Gomes and Paiva (2011).

The stem diameter increase (ΔSD) was obtained through the data collected every 15 days until the end of the experiment (90 days after transplantation).

Possible differences between treatments were verified by analysis of variance (ANOVA). The variables that exhibited significant differences were submitted to regression analysis in order to verify the plants growth response in proportion to the CRF increasing doses in both growing environments. The data analysis was performed in the statistical package Sisvar (Ferreira, 2011).

3. Results and Discussion

At the end of the experiment (90 days after transplantation), the survival rate of the peach rootstock plants was of 100% for all treatments. All variables exhibited interaction ($p < 0.05$) between the environment factors and the CRF (Osmocote®) doses (Table 3), indicating that the study of factor interaction is important to define the best condition to stimulate plant growth and development.

Table 3 - Summary of the variance analysis for contrasts between the environmental factors of cultivation and CRF (Osmocote®) doses for the variables stem diameter (SD), plant height (H), number of leaves per plant (NL), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), plant height and stem diameter (H/SD) ratio, and the Dickson Quality Index (DQI) of peach trees cv. Capdeboscq 90 days after transplantation

Source of variation	df	Mean square							
		SD (mm)	H (cm)	NL	SDM (g)	RDM (g)	TDM (g)	H/SD	DQI
Environment (E)	1	128.2 **	1897.8 **	56.2 **	603.8 **	96.9 **	216.2 **	474.4 **	42.0 **
Dose of CRF (D)	3	137.7 **	549.8 **	109.8 **	233.8 **	37.9 **	84.1 **	103.0 **	60.7 **
E x D	3	8.0 **	162.9 **	11.6 **	70.1 **	12.6 **	26.9 **	31.4 **	3.9 *
Mean		4.3	60.8	55.0	5.9	9.6	15.5	13.4	1.7
CV (%)		4.0	4.3	11.9	10.1	25.5	17.0	6.4	9.7
Mean Greenhouse		4.6	80.9	64.0	8.48	13.8	22.3	16.7	1.8
Mean Open air		3.9	40.7	46.0	3.3	5.3	8.6	10.0	1.5

* Significant at the probability level ($p<0.01$) and ** significant at the probability level ($p<0.05$) by the F test.

All morphological variables exhibited a quadratic behavior in the adjustment of the regression equations (Figs. 1, 2, 3 and 4), proving that the highest dose test results decreases the variables values, that is, increasing the fertilizer dosage allows the increase of the plants growth up to the maximum technical efficiency dose (MTED).

The MTED for plant height in the protected environment was of 6.43 g L^{-1}, corresponding to the height of 113.8 cm, which was three times higher than the control treatment (substrate without the addition of Osmocote®) in the same environment, 90 days after transplantation (Fig. 1). The MTED for plant height in the external environment was of 5.09 g L^{-1}, whose plants reached the height of 52.1 cm, in contrast to the 23.9 cm high of the treatment without the addition of CRF (Fig. 1).

Similar height growth was also observed with the application of Osmocote® in the studies performed by Silva *et al.* (2011) in the production of Rangpur lime rootstock [*Citrus limonia* L. (Osbeck)] and by Dutra *et al.* (2016) in the growth of Canafistula [*Peltophorum dubium* (Spreng.) Taub.]. However, the environments and doses tested in their studies were different of this one.

The positive effects of CRF application on plant growth in different species reinforce the necessity of specific studies to enable the definition of the MTED for each species and cultivar, which may provide superior growth and efficiency in the use and exploitation of fertilizers by the plants.

The estimated MTED for the SD variable of the plants cultivated in greenhouse was of 7.29 g L^{-1}, corresponding to a diameter of 5.51 mm (Fig. 2). According to the current legislation of the Secretary of Agriculture and Food of Rio Grande do Sul

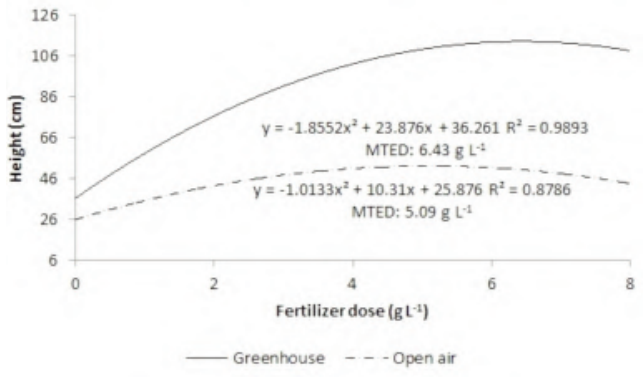

Fig. 1 - Plant height (cm) of the peach plants cv. Capdeboscq, in relation to the dosage of the controlled-release fertilizer (CRF) and two cultivation environments, 90 days after transplanting.

(Ordinance 302/98), the grafting must be performed when the rootstocks reach a SD over 5 mm and a height of 10 cm from the ground. Thus, the MTED estimated for plant grown in greenhouse allows the rootstocks to reach the minimum diameter for grafting at 90 days after transplantation.

On the other hand, in the external environment, the plants have not reach the minimum stem diameter required for grafting during the experiment, even at the highest CRF dose (Fig. 2).

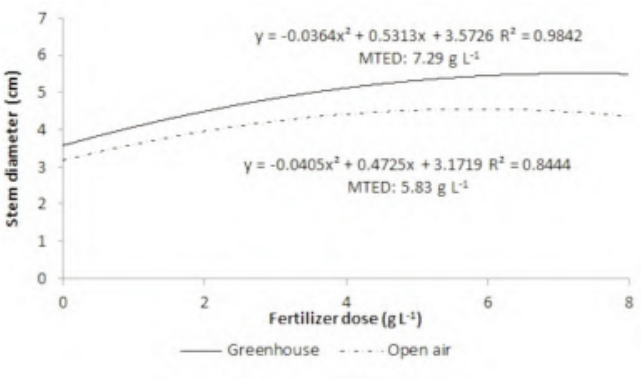

Fig. 2 - Stem diameter (mm) of peach plants cv. Capdeboscq, in relation to the dosage of the controlled-release fertilizer (CRF) and two cultivation environments, 90 days after transplanting.

In peach rootstock production, it is extremely important to define the environment and the fertilizer MTED to obtain the ideal SD for grafting in a shorter period. The minimum diameter of 5 mm is used to make sure that the rootstocks phloem and xylem have greater competence to perform rapid vascular connection between the scion/rootstock (Santarosa *et al.,* 2016), which will allow the effective translocation of the nutrients absorbed by the roots (rootstock) to the aerial part (scion cultivar). This condition will result in a higher percentage of graft-take and growth of the grafted plants, and it will reduce the period to obtain commercial plants.

Souza *et al.* (2013) stress the influence of the rootstock diameter to reduce the time to develop grafted plants of "Ponkan" tangerine. The plants rapid growth was obtained with the use of rootstocks with larger diameter in the appropriate cultivation conditions in the greenhouse.

The best plant growth in both height and stem diameter was obtained in plants grown in a greenhouse with the MTED. This suggests that the greenhouse environment provided better conditions of temperature, humidity and luminosity for the plant growth. Associated with the MTED, these conditions

allowed the plants to reach the ideal point for rapid grafting, which is a desired aspect in the production system of peach tree rootstocks.

Paricá seedlings [*Schizolobium amazonicum* Huber ex Ducke] grown in a protected environment also exhibited superior performance for plant height and stem diameter (Frigotto *et al.,* 2015). These authors concluded that greenhouse cultivation significantly increases the growth variable values in comparison with external environment cultivation.

The highest mean number of leaves per plant (97) was obtained with the estimated MTED (4.69 g L^{-1}) for the plants grown in the greenhouse (Fig. 3A).

As for the variable number of leaves, it was found that the plants of cv. Capdeboscq cultivated in an open sky achieved DMET higher than plants kept in a greenhouse (Fig. 3), a fact that may be related to phytosanitary problems, such as, for example, small leaf spots and necroses detected in the leaves of this treatment, during the conduction of the plant experiment. These leaf damage possibly induced damage to the leaf photosynthetic apparatus, however, this damage may have been efficiently reversed through the emission and growth of new leaves.

This hypothesis can be supported by the fact that, at this moment, the plant enhances the production of photoassimilates and destines most of it, the maintenance and maximization of the aerial part, making this organ the drain of greater energy demand, both to stimulate the leaf growth maximizing the capture of light, as well as to boost the thickening of the stem diameter ensuring the robustness of the rootstock.

It should also be noted, according to the results obtained in this work, that plants grown in the open suggest that they have prioritized the increase in the diameter of the stem at the expense of growth in height, as shown in Table 4, a strategy that can increase the robustness of the plants and decrease the exposure of the aerial part, guaranteeing their survival for a longer period, as well as the maintenance of the physiological processes in this cultivation environment, which expose the plants to sudden environmental variations.

The cultivation environment has a strong influence on environmental conditions, such as temperature and global radiation, parameters that are indirectly related to the efficiency of plants in terms of light absorption capacity, and later conversion to energy, as well as in the absorption and use of nutrients.

A gradual production increase of shoot dry matter, root dry matter and total dry matter up to the CRF MTED was registered (Fig. 3B, 3C and 3D), regardless of the cultivation environment. However, the plants grown in greenhouse presented higher values for total dry matter. These results corroborate the effects of Osmocote® in the growth of Rangpur lime rootstocks, as reported by Scivittaro *et al.*

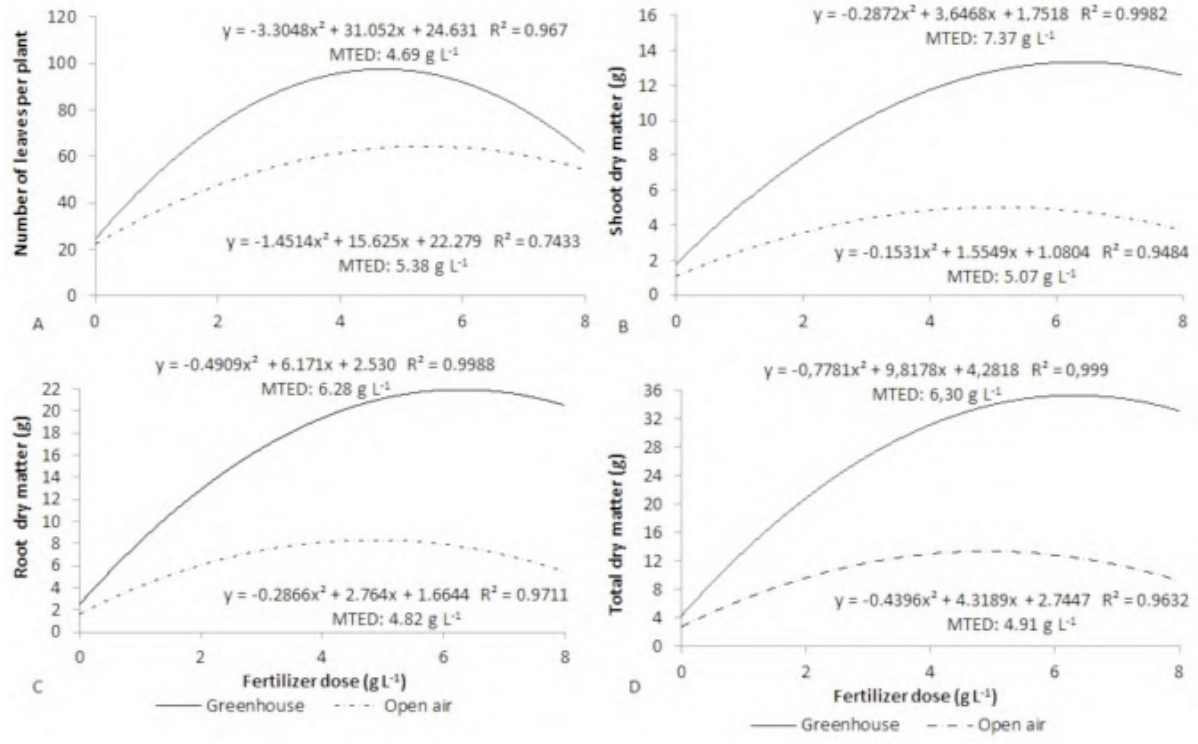

Fig. 3 - Number of leaves per plant (3A), shoot dry mass (3B), root dry mass (3C) and total dry mass (3D) of peach plants cv. Capdeboscq, in relation to the dosage of CRF and two cultivation environments.

Table 4 - Mean values of the differences between greenhouse and open air for the variables stem diameter (SD), plant height (H), number of leaves per plant (NL), shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), plant height and stem diameter (H/SD) ratio, and Dickson quality index (DQI) of peach plants cv. Capdeboscq 90 days after transplantation

Dose	SD (mm)	H (cm)	NL	SDM (g)	RDM (g)	TDM (g)	H/SD	DQI
0	0.47	10.6	4.6	0.7	1.2	1.9	1.9	0.1
2	0.35	33.4	19.8	4.1	5.8	9.9	6.3	0.2
4	0.84	51.6	38.9	7.0	11.9	19.0	8.4	0.5
8	1.11	64.9	6.4	8.7	14.9	23.7	9.7	0.5

(2004). They found that as controlled-released fertilizer doses increased, the dry matter production of the Rangpur lime rootstocks increased as well.

The leaf area has not been quantified in this study. However, there are previous studies that support the increase of the number of leaves per plant is directly proportional to the growth of the leaf area (Menegatti *et al.,* 2017 a). The greatest leaf area of plants grown in greenhouses provides greater efficiency in solar energy uptake for photosynthesis and photoassimilate production, which is directly related to the nutrient supply, including nitrogen (N), present in the CRF formulation used in this study.

The use of CRF in the MTED ensures the availability and efficient utilization of N by the plants because the leaching level of N is reduced in comparison with conventional fertilizers (Zamunér Filho *et al.,* 2012; Muniz e*t al.,* 2013). N is an essential element to the components of the photosynthetic system, such as chlorophylls, carboxylase activity/oxygenase of ribulose 1.5-bisphosphate and carboxylase of phosphoenolpyruvate (Bassi *et al.,* 2018), thus maintaining satisfactory rates of carbon assimilation (Taiz and Zeiger, 2017) and consequently guaranteeing the production of photoassimilates that support plant growth.

The CRF effects on the production of Rangpur lime plants for use as rootstocks were registered by Serrano *et al.* (2006) and Silva *et al.* (2011). They concluded that the fertilizer MTED increases the variables shoot dry matter, root dry matter and total dry matter, stressing the importance of fertilization for the maintenance of the photosynthetic process in order to increase the total dry matter and consequently the plants growth.

The relation between plant height and stem diameter (Fig. 4A) presents a balanced growth of the plants raised in greenhouses.

The H/SD ratio is one of the parameters most used in the plant's quality evaluation. Moreover, it

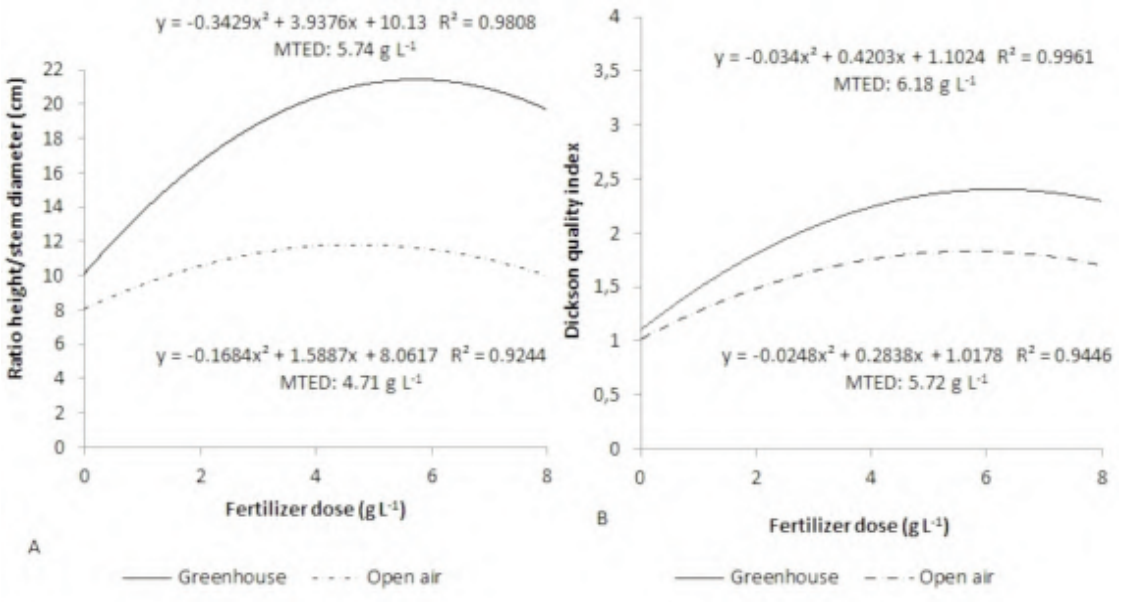

Fig. 4 - Ratio between height and stem diameter and Dickson quality index of peach plants cv. Capdeboscq, in relation to the dosage of controlled-release fertilizer and two cultivation environments.

reflects the accumulation of reserves and ensures greater resistance and adequate potential of the rootstock (Souza *et al.,* 2013). The index considers two parameters in a single indicator, and it can be used as a guide to determine the quality of the plants.

The balance of the plants growth confirmed by the H/SD ratio may have been favored by the use of CRF. The encapsulated fertilizers, such as CRF, allow the slow release of nutrients through a porous structure (Serrano *et al.,* 2006), becoming available to the plants root system over time and according to their nutritional need, avoiding the leach and loss observed in conventional fertilizers.

In addition to the H/SD ratio, the DQI is a good indicator of plant quality. For its calculation, it considers the plants robustness and biomass distribution balance. Therefore, the higher the value, the better the quality standard of the plants will be (Gomes and Paiva, 2011).

The ideal value considered for the DQI is approximately of 2.00 (Gomes and Paiva, 2011). In our study, the highest DQI was close to 2.2 for the estimated MTED of 6.18 g L^{-1} in plants grown in a greenhouse. However, for the plants kept in the external environment, the highest DQI value was of 1.83 with an MTED of 5.72 g L^{-1}, which is lower than the ideal value (Fig. 4B).

Previous research from Dutra *et al.* (2016) and Zamunér Filho *et al.* (2012), in addition to the results obtained in the present study, agree with the results for all morphological variables evaluated. A quadratic positive response was obtained proportionally to the increase of the CRF doses up to the MTED (of approximately 6.2 g L^{-1}). It proves that the plant will not have higher responses if a dose higher than the MTED is applied.

The ΔSD, evaluated every 15 days after the beginning the experiment, is presented in figure 5. For the plants cultivated in a greenhouse, the use of the doses of 4 and 8 g L^{-1} of Osmocote®, incorporated into the substrate, were proven efficient for the production of rootstocks suitable for grafting after 90 days because they presented a final SD mean of 5.1 mm and 5.5 mm, respectively.

Considering the aforementioned results and the relevance of the SD variable in the production of peach rootstocks, we suggest the incorporation of at least 4 g L^{-1} of Osmocote® into the commercial substrate and the cultivation of the plants in a greenhouse. Those conditions can increase the efficiency of the fertilizer use to obtain plants that can be grafted after 90 days.

The effects of different environments on the production of peach rootstocks cv. Okinawa were reported by Reis *et al.* (2010). They reached the ideal point for grafting after 179 days in a protected environment. Schmitz *et al.* (2014), evaluating the production of peach rootstocks cvs. Capdeboscq and Okinawa, in three production systems, reached the grafting point after 154 days.

Fischer *et al.* (2016), researching the influence of the stratification period on wet cold in the emergence and production of several peach rootstocks in the field, obtained materials suitable for grafting after 240 days. These results indicate that the use of CRF and a protected environment are promising in the shortening of the productive cycle, as the grafting

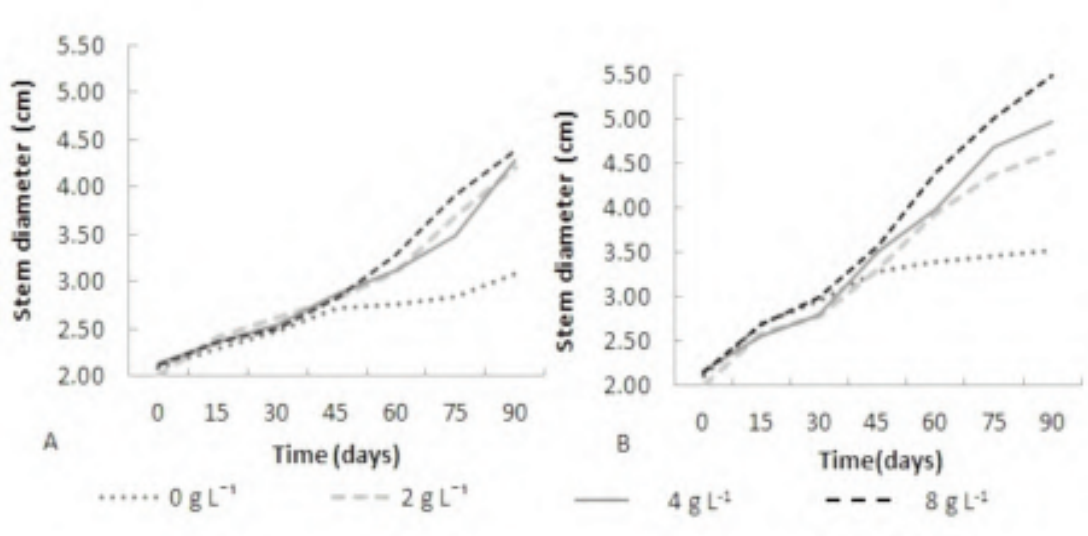

Fig. 5 - Increase of stem diameter, over time, of peach plants cv. Capdeboscq, in relation to the dose of the CRF and two cultivation environments (A) Open air and (B) Greenhouse.

point can be reached after 90 days. This outcome is probably due to the ready availability of nutrients and the maintenance of the plants under favorable environmental conditions, although these results must be validated for other cultivars of [*Prunus persica* L. Batsch] rootstocks with potential use in Brazil, such as cvs. Flordaguard, Okinawa and the Tsukuba series (Menegatti *et al.,* 2019 a).

The greatest ΔSD occurred between 30 and 60 days after transplantation (Fig. 5), regardless of the dose employed, which may be due to the slow releasing of the nutrients, a main characteristic of the fertilizer used, which resulted in the greater amount of nutrients available to the plants during the experiment (Huett and Gogel, 2000). This result also suggests that it is necessary to replace the mineral elements around 60 days after the first application in order to maintain the plants optimal growth rate. Therefore, studies to elucidate the best CRF replacement period are necessary in order to ensure continued growth through the plants different development stages.

The CRF used in the production of peach rootstocks proved to be a promising alternative in comparison with conventional fertilizers. The CRF slowly and continuously releases nutrients to the plant, avoiding leach losses and volatilization. Furthermore, it ensures a better use of nutrients and reduces the environmental and economic impact (especially by the nitrogen economy, which is an expensive and easily leachable element that has a great potential to pollute the environment).

The negative aspect of using CRF is the higher cost in comparison with conventional fertilizers. However, the application of the MTED, with the purpose of maximizing the input use in the production of rootstocks, has been proving to be an economically viable alternative if we consider the price increase of basic inputs. Other characteristics to be considered are the conventional fertilizers high susceptibility of leaching due to the frequency of irrigation in the nursery and the need for parcelled applications, which are driven by higher production costs (Melo Júnior *et al.,* 2014).

The lowest ΔSD presented by plants that are grown in full sunlight (external environment) can be attributed to the restriction of the ideal microclimatic conditions for the plants growth, such as solar radiation, precipitation, wind and temperature. In our region, the high temperatures at full sun, which occur between October and January, may have caused thermal stress. Such conditions reduce transpiration, which consequently decreases the photosynthetic rates and accelerates the respiratory metabolism, reducing the growth rate not only for SD, but for all morphological variables (Afonso *et al.,* 2017; Bassi *et al.,* 2018).

Another factor that may have contributed to reduce the plants growth in external environment was the CRF formulation. The granules contain a homogeneous combination of nutrients and are covered by an organic resin that regulates the nutrients release proportionally to the substrates temperature and humidity (Melo Júnior *et al.,* 2014). In addition to the high temperatures, the heavy rainfall can contribute to accelerate the release of CRF nutrients, resulting in leaching losses.

According to the data provided by the Agroclimatology Station of Pelotas (EAPEL, 2017), between October 2016 and January 2017, the cumulative rainfall reached 549 mm, with a monthly average of approximately 137 mm, concentrated in three to four days of each month. The intense and concentrated precipitation in a short period of time may have caused greater leaching of the nutrients present in the soil solution that had the plants in external environment, reducing the efficiency of the fertilizer use and resulting in lower values for the evaluated variables in comparison with the plants grown in a greenhouse, which did not suffer the influence of the precipitation variable.

Considering the results obtained in this study, it was suggested that the control of the environment for plant cultivation provides greater efficiency in the use of productive resources (nutrients, water, temperature, light and others). In addition to these factors, the use of CRF incorporated into the substrate contributes to cause precocity in the production of peach rootstocks (reduction of the period to reach the grafting point) with a high-quality standard.

4. Conclusions

Considering the results obtained in this study, the cultivation of plants in a greenhouse is proposed, since it provides the best conditions for the use of CRF by the plants, and the concomitant use of the minimum dose of 4.0 g L^{-1} because it reduces the period to reach the grafting point to 90 days.

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Effects of putrescine application in culture medium in improving chamomile [*Chamomilla recutita* (L.) Rauschert.] tolerance to osmotic stress under *in vitro* conditions

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Abstract: In order to assess the effect of osmotic stress induced mannitol under *in vitro* conditions on some growth parameters of chamomile [*Chamomilla recutita* (L.) Rauschert], treatments were arranged and compared for the main effect of osmotic stress induced by mannitol in four levels (0, 2, 4, and 6 g/l), and the interaction effect of osmotic stress x putrescine (0, 0.5, and 1 mM). Osmotic stress, especially induced by 4 and 6 g/l of mannitol, were found to significantly reduce shoot height, root length, the number of shoot and root per plant, the fresh weight of shoot, and the fresh weight of root. When plants were grown on 0.5 mM of putrescine, the fresh weight of root, carotenoid, chlorophyll a, and chlorophyll b were increased, compared to plants grown on medium with 0 and 1 mM of putrescine. Plants grown on medium with 0.5 and 1 mM of putrescine had an increased level of flavonoid, phenolic acid, and proline under four levels of mannitol. The amount of 0.5 mM of putrescine significantly improved plant biomass and essential oil content in plants grown on medium containing 0 and 2 g/l of mannitol. The results showed that the use of putrescine could improve chamomile tolerance to osmotic stress.

1. Introduction

Chamomile [*Chamomilla recutita* (L.) Rauschert.] is an important medicinal plant, believed to have many properties. Several studied have indicated the medicinal effects of chamomile on many diseases (reviewed in Singh *et al.*, 2011). With the growing importance of chamomile applications in modern medicine, many studies have focused on the investigation of exogenous factors such as plant growth regulators and environmental stresses on the growth parameters and physiological characteristics. It has been suggested

that the medicinal properties of chamomile result from its essential oil and antioxidant content (Edris, 2007; Wei and Shibamoto, 2007; Roby *et al.*, 2013). However, these properties are negatively affected by environmental factors such as osmotic stress (Baghalian *et al.*, 2011; Jeshni *et al.*, 2017). A study on drought effects on physiological and phytochemical traits of chamomile reported that agro-morphological characters, essential oil content and composition are significantly decreased in this condition (Baghalian *et al.*, 2011). Afzali *et al.* (2006) showed that osmotic stress induced by polyethylene glycol and mannitol decreases the growth parameters at early growth stages of chamomile.

Polyamines have been found to involve in plant response to biotic/abiotic stress including osmotic stress, and other types of stress such as drought and salinity which impose osmotic stress on plants rather than their own specific effects (Alcázar *et al.*, 2010; Shabala and Munns, 2017; Shokri-Gharelo and Noparvar, 2018). It has been shown that high levels of polyamines in plants are associated with tolerance to abiotic stress (Alcázar *et al.*, 2010; Mandal *et al.*, 2014; Pál *et al.*, 2018). It has also been shown that exogenous application of polyamines, including putrescine, increases the tolerance of plants to stressful conditions (Talaat *et al.*, 2005; Bibi *et al.*, 2010; Hassanein *et al.*, 2013). Exogenous application of putrescine has been shown to improve morphological parameters (plant height, root length, number of shoots and roots, and plants biomass), physiological characters, and phytochemical properties in wheat (Mostafa *et al.*, 2010), geranium (Ayad *et al*., 2010), and Egyptian carnation (El-Ghorab *et al.*, 2006). Furthermore, positive effects of exogenous putrescine application have been shown to improve growth characters and tolerance under abiotic stress (Ali *et al.*, 2007; Bibi *et al.*, 2010; Hassanein *et al.*, 2013; Mandal *et al.*, 2014).

There is no published evidence on the application of putrescine *in vitro* culture medium and its effects on morphological, physiological, and essential oil content of chamomile under different levels of osmotic stress induced by mannitol. The objectives of this study were therefore to investigate the effect of osmotic stress induced by mannitol under *in vitro* conditions and to evaluate the effects of putrescine on ameliorating the negative effects of osmotic stress on chamomile.

2. Materials and Methods

Plant materials and experimental conditions

Seeds of German chamomile [*Chamomilla recutita* (L.) Rauschert.] were used in this work. The experiment was carried out under *in vitro* conditions. Test tubes were used as the experimental unit and one plant was cultured in each test tube. All test tubes used in the experiments were sterilized. Plants were grown in a growth room with temperature 25±2°C, relative humidity 50% and 60% during day and night respectively, and 14h photoperiod throughout the experiment.

In order to measure the main effects of osmotic stress on chamomile and the effects of putrescine application in culture medium in reducing the stress effects, two experiments were designed with the same laboratory conditions. The first was based on a completely randomized design with one factor with four levels (osmotic stress). The second was arranged in factorial design based on completely randomized design (4 x 3) with two factors. Four replications were used in both experiments, and 16 units for the first experiment and 48 for the second experiment were analyzed.

Medium culture and experiments

Basic MS (Murashige and Skoog, 1962) was used as the culture medium. Seeds of chamomile were sterilized in a commercial chlorine solution (5%) for 20 minutes and then washed three times using distilled water. Seeds were then gently placed on culture media.

In the first experiment, one treatment including mannitol in four levels (control, 2, 4, and 6 g/l in culture media) was studied. In the second experiments, two treatments were studied; four mannitol levels were used, 0 (control), 2, 4, and 6 g/l in culture media to create osmotic stress combined with three levels of putrescine 0 (control), 0.5, and 1 mM.

Morphological traits

The morphological parameters of chamomile measured in this experiment were following, shoot height (cm), root length (cm), shoot (n/plant), root (n/plant), fresh weight of root (RFW g/plant), and plant biomass (%). Plant height was measured from the crown to the tip of the stem. Fresh root was carefully washed with tap water after harvest and measured from the crown to the tip of the main root. To measure dry weight, plants were dried in an oven with 72°C temperature for 72 hours.

Chlorophyll content (a and b) and carotenoid

The amount of 0.2 g of fresh leaves were ground in 10 mL of 99% methanol, then centrifuged at 3000 rpm for 5 min. The extract was used to measure light absorption at 653, 470, and 666 nm (Lutts *et al.*,

1996). The following equations were used for calculating chlorophyll content:

CHLa=chlorophyll a= 15.65 A666-7.34 A653
CHLb=chlorophyll b= 27.05 A653-11.21 A666
Cx+c=carotenoid=1000 A470 -2.860 CHLa- 129.2CHLb

Measurement of flavonoid and phenolic acid

The semi-dried samples were solved in 0.1 mol/l sodium acetate at 20:1 ratio (liquid: sample) at room temperature. The mixture was homogenized and centrifuged at 20000 g for 30 minutes at 4°C. The supernatant were aspirated and used to determine flavonoid and phenolic acid content. The procedure described by Zhishen *et al.* (1999) was followed to measure flavonoid. The sample mixed with a solution containing aluminium chloride and sodium nitrite was added to 30 µl of sodium nitrite (10%), 60 µl of aluminium chloride hexahydrate (20%), 200 µl of NaOH (1M) and 400 µl of water. The absorbance reading was recorded at 510 nm every 20 s for 1 minute. The absorbance reading was compared to a standard curve drawn from catechin (69-689 µmol/l). The data were expressed as µmol catechin equivalents per gram of fresh or dry matter.

To measure phenolic acid, 2.5 mL of the Folin-Ciocalteu reagent and 2 ml of saturated sodium carbonate (75 g/L) were mixed with 50 µl of sample and homogenized for 10 s and heated for 30 minutes at 45°C. The absorbance reading was recorded at 720 nm and compared to the standard curve made from gallic acid (235-1176 µmol/l). The data were expressed as µmol gallic acid equivalents per gram of fresh or dry matter.

Proline content

To determine proline content of shoot, 0.5 g of the sample were homogenized in 3% (w/v) sulphosalycylic acid and then filtered through filter paper (Bates *et al.*, 1973). Acid ninhydrin and glacial acetic acid were added into the mixture and then heated at 100°C for 1 h in a water bath. Toluene was used to extract the mixture and the absorbance of fraction was read at 520 nm. Proline concentration was determined using calibration curves and expressed as µmol proline g FW.

Essential oil content

Hydrodestillation was used for the extraction of essential oil, where the sample of 25 g of chamomile herb dried in an oven was homogenized and boiled in 600 mL of distilled water in Clevenger for 3 hours. Then, water was gently removed from the tank and the amount of extracted essential oil was measured.

Statistical analysis

Three weeks after culturing, the data were analyzed by one-way and two-way ANOVA using JMP8-Statistics Software. Mean values were separated with Duncan's multiple range test (P≤0.05).

3. Results

Effect of osmotic stress on growth parameters

The statistical analysis of data from first experiment (16 experimental units) showed that osmotic stress had significant effects on shoot height and fresh weight of shoot at P<0.01, and on root length, number of shoots, number of roots, and fresh weight of roots at P<0.05. The main effect of osmotic stress on morphological traits is shown in figure 1, with an evident reduction in morphological traits under M2, M4, and M6. Mean comparison of data showed that morphological traits decrease with increasing levels of osmotic stress. Control plant (without stress) showed the highest morphological traits compared to plants grown under M2, M4, and M6. Plants grown on medium with 6 g/l of mannitol showed significant reduction (Fig 1B). The main effect of osmotic stress at M2 and M4 levels was more adverse on shoots than on roots. Root length, number of roots, and fresh weight of root were significantly decreased under M2 and M4 compared to a control group according to Duncan's multiple-range test (P≤0.05), but no significant difference was observed between plants grown under M2 and M4 conditions (Fig. 1B). The number of shoots showed a significant decrease, and plants which were grown on culture medium containing 2 and 4 g/l of mannitol, had no significant difference (P≤0.05) (Fig. 1B). Under M6 conditions, all growth parameters especially shoot traits showed sever reduction.

Effect of putrescine application on morphological traits under osmotic stress

The second experiment compared two treatments including osmotic stress and application of putrescine (a total of 48 experimental units). Variance analysis of data revealed that interaction effects of osmotic stress and putrescine (OS x Pu) were significant for shoot length, root length, number of shoots, and fresh weight of shoots (Table 1), while the main effect of putrescine was a significant on the fresh weight of roots (Fig. 2). The Interaction effect and the main effect of treatments were not significant on number of roots per plant (Data not shown).

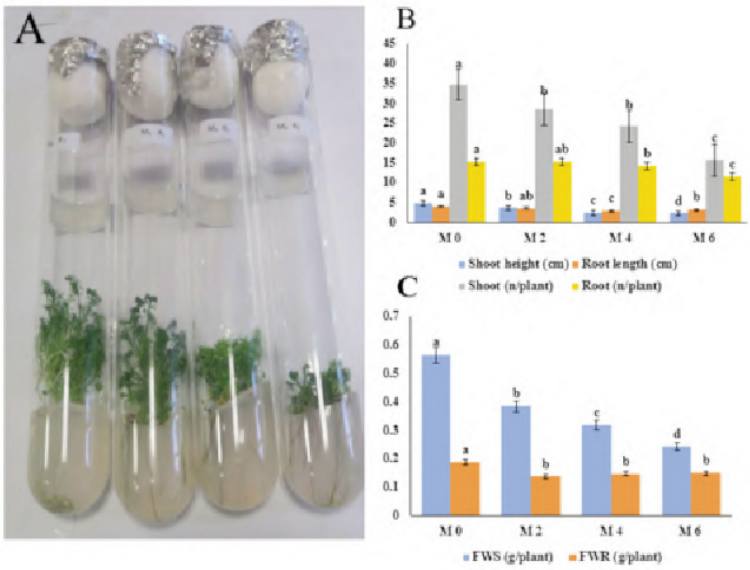

Fig. 1 - Main effects of osmotic stress induced by mannitol (M0= without mannitol, M2= 2 g/l of mannitol, M4= 4 g/l of mannitol, and M6= 6 g/l of mannitol) in culture medium on morphological traits of chamomile. (A) Morphological traits under different levels of osmotic stress (M0= without mannitol, M2= 2 g/l of mannitol, M4= 4 g/l of mannitol, and M6= 6 g/l of mannitol), (B) Mean values of shoot height, root length, number of shoots, and number of roots, (C) Mean values of fresh weight of shoots and roots. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05). FWS= fresh weight of shoot, FWR= fresh weight of root.

Table 1 - Interaction effects of osmotic stress induced by mannitol and putrescine on morphological and chemical traits of chamomile)

Treatment		Morphological traits				Chemical traits		
Mannitol (g/l)	Putrescine (mM)	Shoot length (cm)	Root length (cm)	Shoot (no./plant)	SFW	Flavonoid	Phenolic acid	Proline
M0	Pu0	3.55 b	4.425 b	33.5 a	0.6168 b	33.61 cde	32.87 cde	10.65 d
	Pu0.5	6.025 a	6.325 a	37.5 a	1.615 a	42.02 c	43.24 c	12.86 cd
	Pu1	5.6 a	6.075 a	35.5 a	1.357 a	36.35 cde	34.47 cd	12.26 d
M2	Pu0	2.1 cde	3.275 c	23 b	0.34 bcd	38.38 cd	32.5 cde	16.67 bc
	Pu0.5	2.475 cd	4 bc	24 b	0.5138 bc	22.69 e	17.7 e	15.99 bc
	Pu1	1.6 ef	2.15 e	15 b	0.323 bcd	25.67 de	22.72 de	15.27 bc
M4	Pu0	1.925 def	3.325 c	14.75 cd	0.126 d	70.08 b	62.32 b	18.41 bc
	Pu0.5	2.775 c	3.45 c	19.25 bc	0.4565 bcd	36.77 cde	30.6 cde	19.27 b
	Pu1	1.1 f	1.275 f	8.25 e	0.1505 d	45.11 c	44.76 c	21.46 ab
M6	Pu0	1.125 f	2.3 de	11.5 de	0.2212 cd	71.68 b	67.52 b	24.21 a
	Pu0.5	1.9 def	3.125 cd	17.25 c	0.5102 bc	69.89 b	62.81 b	20.92 ab
	Pu1	1.3 ef	1.175 f	10.75 de	0.193 cd	90.01 a	90.26 a	20.66 ab
Significance								
OS		**	**	**	**	**	**	**
Pu		**	**	**	**	*	*	*
OS x Pu		**	**	*	**	**	**	**

Different letters within each column indicate significant differences according to Duncan's multiple-range test (P≤0.05).
* P<0.05 and **0.01, indicate level of significance.
OS= osmotic stress; M0= without mannitol; M2= 2 g/l of mannitol; M4= 4 g/l of mannitol; M6= 6 g/l of mannitol; Pu0= without putrescine; Pu0.5= 0.5 mM of putrescine; Pu1= 1 mM of putrescine; SFW= fresh weight of shoot.

Putrescine significantly increased the fresh weight of roots at 0.5 and 1 mM compared with plants grown on basic MS medium (without putrescine). In terms of shoot length, root length, number of shoots, and fresh weight of shoots, plants grown on a basic MS medium containing 0.5 and 1 mM of putrescine and without mannitol showed significantly increased traits compared to control plants (without mannitol and putrescine) and other groups (Table 1). Plants grown on medium containing 2 and 4 g/l of mannitol

plus 0.5 mM of putrescine (M2Pu0.5 and M4Pu0.5) showed significantly better traits compared to plants grown on medium containing 2 and 4 g/l of mannitol plus 0 and 1 mM of putrescine (M2Pu0, M2Pu1, M4Pu1, and M4Pu1). However, plants grown on medium with 6 g/l of mannitol (severe osmotic stress) and with/without putrescine showed significantly the lowest means compared to other groups (Table 1).

Effect of putrescine application on physiological traits under osmotic stress

Variance analysis of physiological traits showed that main effect of putrescine on carotenoid, chlorophyll a and b was significant (Data not shown), while interaction effect of osmotic stress x putrescine was significant on flavonoid, phenolic acid and proline (Table 1).

The carotenoid, chlorophyll a and b of chamomile were significantly increased in plants grown on medium with 0.5 mM of putrescine (Fig. 2). The flavonoid, phenolic acid, and proline content in plants grown on medium with 1 mM of putrescine plus 6 g/l of mannitol showed the highest content compared to other groups (Table 1). The lowest contents of flavonoid, phenolic acid, and proline in each group (M0, M2, M4, and M6) were observed in plants grown on medium without putrescine (Pu0) and the highest contents in each group were observed in medium with 1 mM of putrescine (M0Pu1, M2Pu1, M4Pu1, and M6Pu1) compared to control groups.

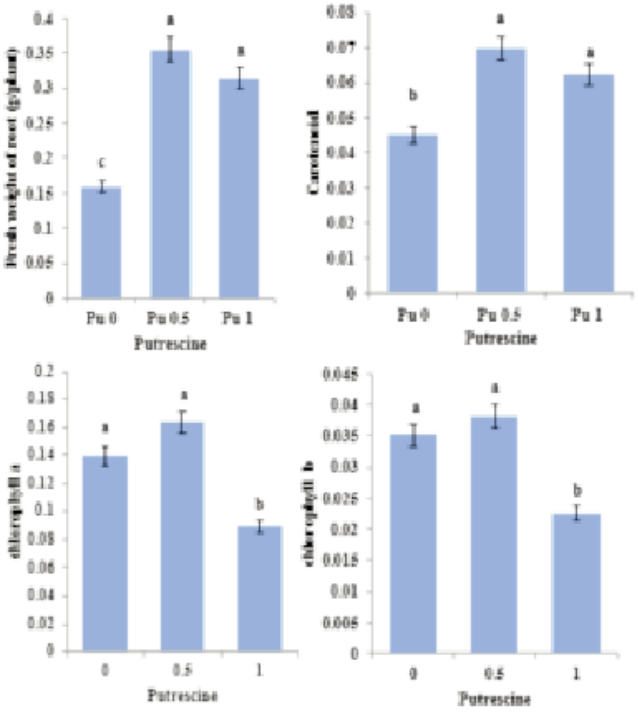

Fig. 2 - The effect of putrescine on morphological and physiological traits of chamomile (fresh weight of root, carotenoid, chlorophyll a and b). Putrescine was added in 0 (control), 0.5, and 1 mM in culture media. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05).

Effect of putrescine application on plant biomass and essential oil under osmotic stress

Variance analysis of data related to plant biomass and essential oil showed that the interaction effect of osmotic stress x putrescine was significant (P<0.05) (Data not shown). Plants grown on medium containing 2, 4, and 6 g/l of mannitol (M2, M4, and M6) plus 0.5 and 1 mM of putrescine (Pu0.5 and Pu1) had the lowest plant biomass and essential oil content compared to the control group (M0Pu0, M0Pu0.5, M0Pu1) (Fig. 3 and 4).

The largest increase in biomass was observed in plants on medium without application of mannitol but treated with 0.5 and 1 mM of putrescine

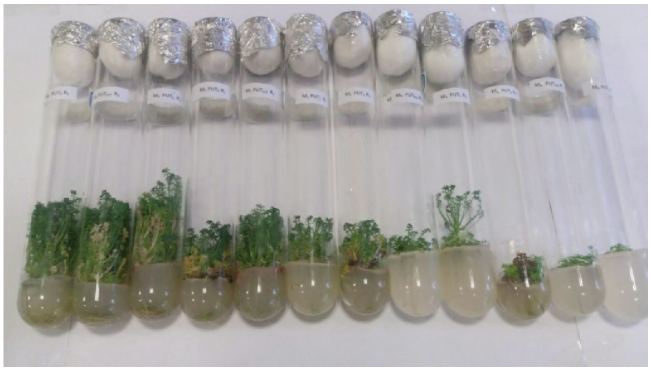

Fig. 3 - Interaction effects of osmotic stress x putrescine on morphological traits of chamomile. Mannitol was used in four levels; 0 (control), 2, 4, and 6 g/l in culture media to create osmotic stress. Putrescine was added in 0 (control), 0.5, and 1 mM in culture media.

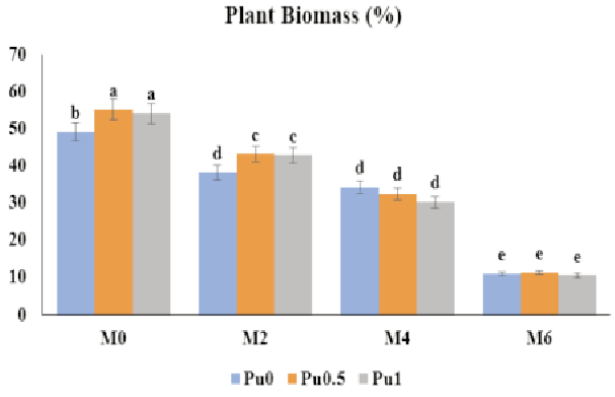

Fig. 4 - Interaction effects of osmotic stress x putrescine on the plant biomass of chamomile. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P ≤ 0.05).

(M0Pu0.5 and M0Pu1). Plants grown on medium with 2 g/l of mannitol plus 0.5 and 1 mM of putrescine had a larger biomass compared to plants grown on M2Pu0, but significantly lower biomass compared to the control group. In plants grown on medium with 4 and 6 g/l of mannitol, no significant difference was observed between plants placed on medium with 0, 0.5, and 1 mM of putrescine (Fig. 3).

The largest amount of essential oil was in the group of plants grown on medium without osmotic stress (M0;) and with 0.5 and 1 mM of putrescine (Pu0.5 and Pu1). The group of plants grown on medium with 2 g/l of mannitol, plants grown on medium with 0.5 and 1 mM of putrescine showed significantly more essential oil (M2Pu0.5 and M2Pu1) compared to plants grown on medium without putrescine (M2Pu0). Even though plants on medium with 0.5 and 1 mM of putrescine plus 4 and 6 g/l of mannitol did not show significant difference compared to plants grown on medium without its application (Fig. 4).

4. Discussion and Conclusions

The effect of putrescine application in culture medium containing mannitol has not previously been reported so far. The studies on other plant species and also other types of stresses will therefore be used for discussion. In this study, the effect of osmotic stress created by mannitol under *in vitro* conditions, the main effect of putrescine on the growth of chamomile, and putrescine effects in ameliorating effects of osmotic stress on chamomile were investigated.

Osmotic stress is a side effect of some abiotic stresses such as drought and salinity in which water absorption is limited, leading to conditions similar to drought (Shen *et al.*, 1999). Drought and salt stress have been found to decrease the morphological traits in many medicinal plants, in terms of length and number of shoots and roots, fresh weight of shoots, and fresh weight of roots (Afzali *et al.*, 2006; Jaleel *et al.*, 2008; Arazmjo *et al.*, 2010; Anjum *et al.*, 2011). In agreement with the findings of this study), Afzali *et al.* (2006) reported a decrease in fresh weight of shoots and roots in chamomile under polyethylene glycol-induced osmotic stress. In another study, Dadkhah (2010) tested the effects of salinity on the plant height and number of shoots of chamomile in a pot experiment. Dadkhah reported a significant decrease in plant height and number of shoots in early stage of the stress. In respect of drought stress induced by mannitol under *in vitro* conditions, Ghaheri *et al.* (2015) findings in *Steviare baudiana* Bertoni are in accordance with the findings of this study.

Regarding putrescine effects in increasing morphological traits, a number of studies have showed that foliar application of putrescine increases plant height, root length, shoot (number per plant), and root (number per plant) (Talaat *et al.*, 2005; Mostafa *et al.*, 2010; Amin *et al.*, 2011; Hassan and Bano, 2016), and improving physiological traits, in terms of chlorophyll a, chlorophyll b, carotenoid (Talaat *et al.*, 2005; Hassan and Bano, 2016), and proline (Hassan and Bano, 2016). This study provides evidence of improved flavonoid, phenolic acid, and other parameters (Table 1, Fig. 2) in chamomiles grown on medium containing 0.5 and 1 mM of putrescine. Studies that investigate effects of putrescine under *in vitro* conditions have not been found, but in agreement with the findings of this study, several studies have revealed that plants treated with putrescine have increased growth parameters and more tolerance to abiotic stresses such as osmotic stress, drought, salinity, and temperature compared to untreated plants (Jaleel *et al.*, 2008; Alcázar *et al.*, 2010; Hassanein *et al.*, 2013). Investigating the effects of putrescine foliar application on chamomile and sweet marjoram under salinity stress, Ali *et al.* (2007) found that putrescine significantly increased flavonoid content. In addition, the use of putrescine was found to enhance chlorophyll a, chlorophyll b, carotenoid, phenolic acid, and morphological traits under stress conditions compared to untreated plants (Amin *et al.*, 2011; Shallan *et al.*, 2012; Hassanein *et al.*, 2013; Hassan and Bano, 2016).

Other authors have indicated positive effects of putrescine application on plant biomass and essential oil in chamomile and sweet marjoram (Ali *et al.*, 2007), wheat (Hassan and Bano, 2016), onion (Amin *et al.*, 2011), and cotton (Shallan *et al.*, 2012). Findings in this study showed that putrescine application improves the plant biomass and essential oil content under normal growth conditions and in chamomiles grown on medium containing 2 g/l of mannitol (Fig. 4 and Fig. 5), whereas plants grown on medium with 4 and 6 g/l of mannitol showed remarkable decreased levels of biomass and essential oil compared to the control group, and the study did not find the positive effects of putrescine under these levels of osmotic stress (M4 and M6). In line with these findings regarding the positive effects of

putrescine under normal growth conditions and 2 g/l of mannitol stress, Ali *et al.* (2007) reported an increase in the plant biomass in *chamomile and sweet marjoram*, Ayad *et al.* (2010) in *geranium* and El-Ghorab *et al.* (2006) in *Egyptian carnation* reported a high level of essential oil in plants treated with putrescine.

Pál *et al.* (2018) in their study on wheat reported that putrescine treatment induces stress-responsive genes that overlap with the genes induced by osmotic stress. They suggested that changes induced by putrescine overlap with changes induced by osmotic stress, and lead to better tolerance in plants treated with putrescine. In another study, Bibi *et al.* (2010) showed that putrescine application significantly increases the endogenous putrescine concentration. They suggested that stress tolerance correlates with an increment of putrescine. The results of the present study do not provide evidence at the molecular level and for the endogenous concentration of putrescine in chamomile but the data showed an increased values of observed traits when putrescine was used in medium culture. As suggested by Mandal *et al.* (2014), polyamines including putrescine alleviate oxidative stress induced by osmotic stress. It is believed that oxidative stress created by being exposed to abiotic stresses such as osmotic stress, drought, and salinity is one of most important reasons for remarkable reduction of morphological traits and plant yield (Shokri-Gharelo and Noparvar, 2018). Other studies have shown that plants with efficient antioxidant systems, including high level of flavonoid, phenolic components (reviewed in Shabala and Munns, 2017), and plants with high level of proline content (Ahmad *et al.*, 2016) show more tolerance and these indices have been regarded as one of tolerance characters in different plants. This study revealed that flavonoid, phenolic acid, and proline are increased in chamomile grown on medium with putrescine (Table 1). This could explain the better morphological and physiological traits as well as essential oil content studied in this work in plants under osmotic stress.

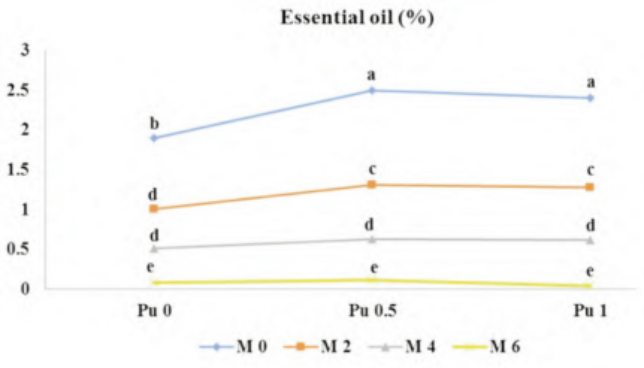

Fig. 5 - Interaction effects of osmotic stress x putrescine on the essential oil of chamomile. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05).

Osmotic stress, especially at M4 and M6 levels (4 and 6 g/l of mannitol) significantly reduce the morphological traits of chamomile under *in vitro* conditions. The main aim of the current study was to use putrescine in culture medium to assess its effects in ameliorating osmotic stress. The values of morphological traits, some physiological traits, and contents of essential oil of chamomile were significantly higher in plants grown on medium containing 0.5 and 1 mM of putrescine compared to plants grown on medium without putrescine application.

Application of putrescine may improve chamomile tolerance to osmotic stress, and may be considered as one of substances that can be used to improve chamomile quality under osmotic stress.

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Pinto bean and black mustard responses to bio-fertilizers under intercropping system

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Key words: black mustard, land equivalent ratio, nitrogen, pinto bean, relative value total, relative yield total.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: In order to evaluate the response of pinto bean and black mustard intercropping to application of biological and chemical nitrogen fertilizers, a factorial set of treatments was arranged within randomized complete block design (RCBD) with three replications. In this experiment, fertilizer treatments were non-fertilizer, bio-fertilizers, bio-fertilizers + 50% chemical urea fertilizer (125 kg/ha) and bio-fertilizers + 100% chemical fertilizer (250 kg/ha). The cropping patterns comprised pure stands of bean and black mustard, additive intercropping with a ratio of 50% black mustard + optimum density of pinto bean mono cultures and an additive intercropping with optimum density of two species in mono cultures. Application of bio-fertilizers and chemical fertilizer increased most of the agronomic traits in pinto bean and black mustard plants. The bio-fertilizers + 100% of urea followed by bio-fertilizers + 50% of urea were the superior treatments, compared with other fertilizers. Evaluation of intercropping patterns with using land equivalent ratio (LER), relative yield total (RYT), relative value total (RVT) and relative crowding coefficient (RCC) indices showed that the highest LER and RYT were recorded for bio-fertilizer + 100% chemical fertilizer treatment. The highest RVT and RCC were obtained from control treatment (non-fertilization) in inter-cropping (optimum density of two species). Based on the LER, RVT, RYT and RCC indices, it was evident that intercropping of pinto bean and black mustard was more beneficial than mono cultures. Therefore, it was generally concluded that intercropping pattern was better than monocultures of two species at different levels of fertilizers and also bio-fertilizers application could increase efficiency of chemical fertilizer. Thus, bio-fertilizers + 100% chemical fertilizer and intercropping of pinto bean and black mustard was the better treatment.

1. Introduction

To increase the efficacy of crop production, improve soil fertility and environmental protection, an alternative cropping system could be needed (Kiminami *et al*., 2010). Intercropping is a method for moving towards sustainable agriculture and environmental protection (Habimana *et al*., 2019; Moghbeli *et al*., 2019). One of the farming practices is concurrent cultivation of two or more crops in the same field which is experienced in

many regions of the world (Tüzel and Öztekin, 2017). Some reasons have been identified for farmers engaging in intercropping which are still valid today. First, it leads to increase in the utilization of environmental factors. This has both space and time dimension.

Plants are different in rooting habitat and have different nutrient requirements. Thus, the intercropping of plants can increase the utilization of nutrients, water and light. Also, intercropping can lead to reduction of adverse conditions in the agroecosystem (Lithourgidis *et al*., 2011). Intercropping may also lead to better soil management because of the fact that may crops overlap in terms of the time they are in the soil. Other economic reasons such as dependability of returns and increased returns from the same piece of land may make farmers adopt intercropping (Alabi and Esobhawan, 2006). Watikai *et al*. (1993) and Willy (1990) confirmed that increasing the yield of biomass in intercropping is due to the more absorption of light. The highest performance is achieved when intercropping canopy is composed of two layers: (1) tall plants with narrow leaves and high photosynthetic capacity; (2) dwarf plants with lying leaves and low photosynthetic capacity. In general, the productivity in intercropping is more than sole cropping (Raei *et al*., 2015).

Among nutrient elements, nitrogen is an important nutrient and has vital functions in plant growth and development. Nitrogen deficiency imposes most limits on crop production compared to other nutrients. With large areas of the arable land in Iran being located in arid and semiarid regions, most of them face low organic matter content as well as nitrogen deficiency and also, to achieve an economically sound production, nitrogen plays a significant role in these regions (Joorabi *et al*., 2015). On the contrary, slow-release nitrogen fertilizers are effective and inexpensive alternative to soluble N (Jiao *et al*., 2005). The yield of pea in intercropping of pea and wheat increased by application of slow release nitrogen fertilizer (Abbady *et al*., 2016). In all around of the globe, for achieved high yield of plants, the chemical fertilizers are extensively being used. However, this type of fertilizers has devastating effects on the health of the soil animals. A better alternative of these chemicals might be to exploit the microbial capabilities to be served as bio-fertilizer (Tomer *et al*., 2016). Bio-fertilizers colonize at the rhizosphere and improve nutrient accessibility of plants and increase the growth of plants. Microorganisms residing in rhizosphere immensely facilitate trace element's uptake. They may act as biocontrol agent, by means of antagonistic activity against phytopathogenic microorganisms, interfering in the bacterial quorum sensing systems, etc. However, bio-fertilizers perform more than one mechanism for accomplishing plant growth enhancement (Kumar *et al*., 2014; Dutta and Patel, 2016).

Black mustard is an important oilseed crop. It is often grown as an intercrop or mixed crop either with pulses or cereals crops, but its productivity is very low due to improper combination (Kumar *et al*., 2014). Bean is also one of the most important food supplements for human, and its protein content is rich (Arija *et al*., 2007). It is also tolerant to shadow and can be planted in intercropping system and grows well. It can increase the soil nitrogen by nitrogen fixation (Kowal and Kassam, 1978). Intercropping of legumes with non-legumes increases yield per unit area, because they use different nitrogen sources and have low competition for nitrogen (Haugard-Nielsan *et al*., 2001). The importance of this pulse crop is based on its good nutritive composition and its high market value, which mainly depends on the consumption quality of the product (nutritional and culinary quality of either the seed or the pod). Thus, the present investigation was carried out to study pinto bean and black mustard responses to bio-fertilizers and chemical nitrogen fertilizer, intercropping system and interaction of intercropping system × nitrogen fertilizer.

2. Materials and Methods

Field conditions

The experiment was conducted in 2016 at the Research Farm of the Faculty of Agriculture, University of Tabriz, Iran (Latitude 38°05' N, Longitude 46°17' E, Altitude 1360 m above sea level with the mean annual rainfall of 285 mm). Some physical and chemical properties of soil in experimental area and averages of maximum and minimum temperatures and rainfall during the work in 2016 were shown in Table 1.

Experimental design and treatments

A factorial set of treatments was arranged with three replications. In this experiment, fertilizer treatments were control (non-fertilizer), bio-fertilizers (azotobarvar 1 and barvar 2), bio-fertilizers + 50% the recommended chemical urea fertilizer (125 kg/ha) and bio-fertilizers + 100% chemical fertilizer (250

Table 1 - Some physical and chemical properties of experimental soil and averages of maximum and minimum temperatures and rainfall during the work in 2016

Physical and chemical properties of experimental soil												
Depth (cm)	EC (ds/m)	PH	Organic Carbon (%)	N (%)	P (mg/kg)	K (mg/kg)	Fe (mg/kg)	Ca (mg/g)	Sand (%)	Silt (%)	Clay (%)	Soil type
0-35	2.77	7.75	0.37	0.04	4.90	255	2.60	780	74	14	12	Sandy loam

Months	Averages of maximum and minimum temperatures and rainfall	
	Temperature (°C)	Rainfall (mm)
April	9.4	78.2
May	16.9	13.5
June	22	14.8
July	28	0
August	29.4	15

kg/ha). Azotobarvar 1 contains the azoto bactervinelandii (strain O4) and barvar 2contains the pantoea agglomerans (strain P5) and pseudomonas putida (strain P13). The cropping patterns comprised pure stands of bean and black mustard, additive intercropping with a ratio of 50% black mustard + optimum density of pinto bean mono cultures and an additive intercropping with optimum density of two species in mono cultures.

Measurements

Yield and yield components. At maturity and when the moisture content of seeds decreased by about 18%, 10 plants were harvested from each plot and 100 grains weight of pinto bean and black mustard were recorded. Also to determine of grain and biological yields, an area equal to 1 m^2 was harvested from middle part of each plot considering marginal effect and dried in an oven at 75°C for 48 hours. Subsequently, biological and grain yields per unit area were determined. Harvest index was calculated by the following equation:

$$\text{Harvest index} = (\text{Grain yield}/\text{Biological yield}) \times 100$$

Evaluative indices of intercropping

Land equivalent ratio (LER), as an agronomic index, indicates the efficiency of intercropping for using the resources of the environment compared with mono cultures (Mead and Willey, 1980). The value of unity is the critical value. When the LER is greater than one, the intercropping improves the growth and yield of the cultivars. In contrast, when LER is lower than one the intercropping negatively affects the growth and yield of the plants grown in mixtures (Caballero *et al*., 1995). The LER was calculated as:

$$LER = \frac{Y_{pb}}{Y_p} + \frac{Y_{bp}}{Y_b}$$

where Y_p and Y_b are the yields of pinto bean and black mustard, respectively, as sole crops and Y_{pb} and Y_{bp} are the yields of pinto bean and black mustard, respectively, as intercrops.

Relative value total (RVT) as an economic index proposed by Schultz *et al.* (1982).

This index is widely used now and has been used by many researchers. The RVT was calculated as:

$$RVT = \frac{aP_1 + bP_2}{aM_i}$$

where, P_1 and P_2 are the yields of two different crops in intercropping and M_1 and M_2 are the yields of those of these crops in monocultures ($M_1 > M_2$). Also, a and b are the market prices of crop 1 and 2 respectively.

If the RVT >1, the mixture crop has the advantage and if the RVT <1, pure stand will have an economic advantage. If RVT =1, then these two methods are not economically advantageous to each other.

Relative yield is the ratio of the species response in the mixture to the species response when grown in monoculture. Relative yield total (RYT) is the total RY of the two associated species, as shown in below:

$$RYT = RYa + RYb$$
$$RY_a = Ya \text{ in mixture } / Ya \text{ in monoculture}$$
$$RY_b = Yb \text{ in mixture } / Yb \text{ in monoculture}$$

A RYT of 1 indicates that species A and B are making demands on the same resources. If RYT is <1, this

shows antagonism between species A and B. If the RYT is >1, the yield of the mixture is greater than that of the single and is preferred.

The Relative Crowding Coefficient (RCC) is a measure of the relative dominance of one species over the other in a mixture (De Wit, 1960). The RCC was calculated as:

$$RCC = (Y_{pb}/Y_p)/(Y_{bp}/Y_b)$$

where Y_p and Y_b are the yields of pinto bean and black mustard, respectively, as sole crops and Y_{pb} and Y_{bp} are the yields of pinto bean and black mustard, respectively, as intercrops.

If RCC= 1, the amount of crop in the mixture will be equal to monocropping. Also, if RCC <1 indicates that the amount of the product in the mixture has decreased relative to solecrop and if RCC >1, the yield of the mixture is higher than that of pure stand of crops and the mixing is beneficial.

Statistical analysis

Analyses of variance for data based on theexperimental design and comparison of means (Duncan multiple range test) at $p \leq 0.05$ were carried out, using MSTATC software. Excel software 2013 was used to draw figures.

3. Results

Analyses of variance showed significant effects of cropping pattern and fertilizers on 100 grains weight, biological and grain yields per unit area of pinto bean and also biological and grain yields per unit area of black mustard. 100 grains weight of black mustard was significantly affected by fertilizer treatments and interaction of cropping pattern × fertilizers (Table 2).

The highest 100 grains weight, biological and grain yields per unit area of pinto bean and grain yield of black mustard were achieved in pure stands of bean and black mustard and also inbio-fertilizers + 100% chemical fertilizer (urea). Maximum biological yield of black mustard was achieved inpure stands of black mustard culture, but there were no significant differences with additive intercropping with optimum density of two species in mono cultures treatment. Also, maximum of this trait was achieved in bio-fertilizers + 100% chemical fertilizer (urea) but, there were no significant differences with bio-fertilizer + 50% chemical fertilizer (Table 3).

Significantly, maximum 100 grains weight of black mustard in different cropping patterns was observed in intercropping with a ratio of 50% black mustard + optimum density of pinto bean mono cultures and bio-fertilizers + 100% chemical fertilizer (urea). Generally, in other cropping patterns there were no considerable differences between fertilizer treatments (Fig. 1).

Evaluation of intercropping efficiency of treatments indicated that land equivalent ratio (LER) is >1 in all intercropping and fertilizer treatments and this showing the superiority of intercropping compared to single cropping. Maximum of LER and relative yield total (RYT) were attended in optimum density of two species and bio-fertilizers + 100% chemical fertilizer (urea). Maximum relative value total (RVT) is related to optimum density of two species with non-fertilizer. Maximum of relative crowding coefficient (RCC) was related to non-fertilizer treatment in 50% of optimum density of two species in pinto bean and optimum density of two species in black mustard (Table 4).

Table 2 - Analysis of variance of the agronomic traits in pinto bean and black mustard under different cropping patterns and fertilizer treatments

Source	df	Mean Square							
		Pinto bean *(Phaseolus vulgaris* L.)				Black mustard (*Brassica nigra* L.)			
		100 grains weight	Biological yield	Grain yield	Harvest index	100 grains weight	Biological yield	Grain yield	Harvest index
Replication	2	72.94	3680597	1131685	2.03	0.01	1786035	157796	2.60
Cropping pattern	2	79.10 **	40310058 **	9924157 **	0.70 NS	0.01 NS	122550980 **	1961700 **	5.77 NS
Fertilizer (F)	3	140.98 **	37336605 **	9970014 **	17.55 NS	0.14 *	24340924 **	832223 **	2.44 NS
C × F	6	4.78 NS	989636 NS	236278 NS	6.49 NS	0.17 **	8957965 NS	11308 NS	6.06 NS
Error	22	2.75	559597	133108	10.29	0.04	5045637	21412	3.77
Cv %	-	5.23	16.85	16.27	6.40	4.46	17.12	8.53	14.59

Ns, * and **: non-significant and significant at p≤*0.05* and p≤*0.01*, respectively.

Table 3 - Means of the agronomic traits in pinto bean and black mustard under different cropping patterns and fertilizer treatments

Treatment	Pinto bean (*Phaseolus vulgaris* L.)			Black mustard (*Brassica nigra* L.)	
	100 grains weight (g)	Biological yield (kg/ha)	Grain yield (kg/ha)	Biological yield (kg/ha)	Grain yield (kg/ha)
Cropping pattern					
C1	34.08± 5.83 a	6505.70± 80.65 a	3269.90± 57.18 a	15181.70± 123.21 a	2066.60± 45.45 a
C2	32.19± 5.67 b	3793.90± 61.59 b	1911.80± 43.72 b	9437.90± 97.14 b	1272.60± 35.67 c
C3	29.00±5.38 b	3013.80± 54.89 c	1543.10± 39.28 c	14737.50±121.39 a	1802.10± 42.45 b
Fertilizer treatments					
F1	26.54±5.15 d	2117.40± 46.01 d	1048.90± 32.38 d	11022.00± 104.98 c	1372.80± 37.05 c
F2	31.23± 5.58 c	3537.50± 59.47 c	1771.20± 42.08 c	12606.00± 112.27	1542.00±39.26 b
F3	33.37± 5.77 b	5138.70± 71.68 b	2688.20± 51.84 b	14266.00± 119.44 a	1959.50± 44.26 a
F4	35.87± 5.98 a	6777.60± 82.32 a	3458.10± 58.80 a	14581.00± 120.75 a	1980.70± 44.50 a

Different letters in each column indicate significant difference at P≤0.05. Means are average values of three replicates ± standard errors.
C1, C2, C3= pure stands of bean and black mustard, additive intercropping with a ratio of 50% black mustard + optimum density of pinto bean mono cultures and additive intercropping with optimum density of two species in mono cultures, respectively.
F1, F2, F3, F4= control (non-fertilizer), bio-fertilizers (azotobarvar 1 and barvar 2), bio-fertilizer + 50% chemical fertilizer (urea) and bio-fertilizers + 100% chemical fertilizer (urea), respectively.

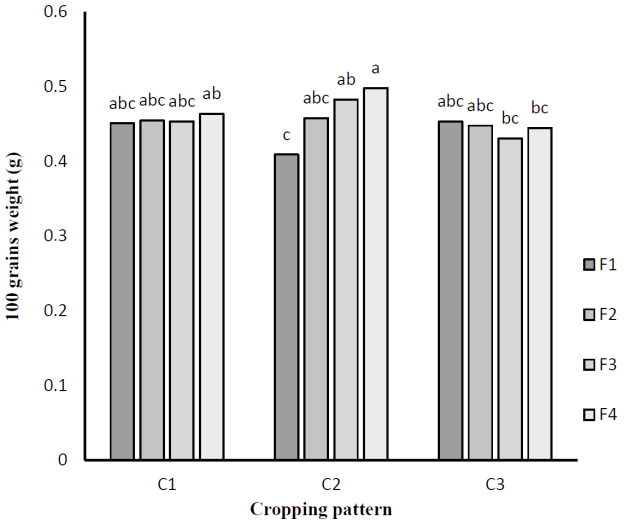

Fig. 1 - Mean 100 grain weight of black mustard for interaction of cropping pattern × fertilizers. Different letters indicate significant difference at p ≤ 0.05 (Duncan test). C1, C2, C3= pure stands of bean and black mustard,additive intercropping with a ratio of 50% black mustard + optimum density of pinto bean mono cultures and additive intercropping with optimum density of two species in mono cultures, respectively. F1, F2, F3, F4= control (non-fertilizer), bio-fertilizers (azotobarvar 1 and barvar 2), bio-fertilizer + 50% chemical fertilizer urea and bio-fertilizers + 100% chemical fertilizer (urea), respectively.

4. Discussion and Conclusions

According to the results, bio-fertilizers + 100% chemical fertilizer (urea) was the best fertilizer treatment in pinto bean and black mustard as, it significantly increased the field performance of these plants (Table 3, Fig. 1), followed by bio-fertilizers + 50% chemical fertilizer. However, biological and grain yields for black mustard was affected as similar to bio-fertilizer + 50% chemical fertilizer (urea) with bio-fertilizers + 100% chemical fertilizer (urea). Therefore, bio-fertilizer application resulted in decreasing 50% of chemical fertilizing. Chemical fertilizer has various negative environmental effects such as soil, water and air pollution, which increase environmental production cost (Moradi *et al*., 2011). Bio-fertilizer as essential components of organic farming, play a vital role in maintaining long term fertility and sustainability of soil. Bio-fertilizers have the ability to access a major part of nutrients for growing plant along with growth promoting factors (Cordovilla *et al*., 1999).

Significant reduction of grain and biological yields in intercropping (Table 3) was attributed to interspecific competition between two crops (Bybee-Finley and Matthew, 2018). Pilbeam *et al*. (1994) has noted that grain yield of maize in sole culture was greater than intercropping with bean. Competition for nutrient uptake and deficiency of nitrogen transport are responsible for the reduction of maize yield in intercropping with legumes (Tomar *et al*., 1988). However, there were not significant differences between sole cropping and optimum density of two species in intercropping system. Therefore, the presence of pinto bean plants hasn't considerable interspecific competition on black mustard plants. Always grain yield of plants did not reduce in intercropping.

As an illustration, Long *et al*. (2001) showed that the grain yield of wheat increased 28 to 30% in intercropping with soybean compared to monoculture.

The land equivalent ratio (LER) of the all intercropping treatments was more than 1, which indicated an advantage of intercropping in comparison with monocultures of pinto bean and black mustard (Table 4). This can be attributed to increasing plant density/m^2 and more use efficiency of environmental resources (Nasrollahzadeh Asl *et al*., 2009). Bio-fertilizers improved LER at all plant population as were applied alone or along with chemical fertilizer. In intercropping system, root interaction could increase the root activity and microbial quantity in the rhizosphere (Zhang, 2013). Interspecific interaction between species in the rhizosphere can also affect nutrient availability and uptake in intercropping (Haugard-Nielsan, 2001). Dua *et al*. (2005) found that intercropping potato and French bean in all intercropping treatments enhanced yield compared to sole cropping and the amount of LER was more than one. Specific competition usually includes competition for soil water, available nutrients, and solar radiation (Buxton and Fales, 1993). Competition can also have a significant impact on the growth rate of the presented species in intercropping.

Relative value total (RVT) of intercropping treatments was higher than 1 which showed the economic advantage of intercropping compared to monocultures. The highest RVT were observed in the non-fertilizerwith optimum densities of two species. RVT was improved as plant density increased. On these biases RVT values of optimum densities for two species were higher than 50% optimum density at the same fertilizer treatments (Table 4). It was attributed to more improvement intercropping yields compared to monocultures (Javanmard *et al*., 2018). Several indices such as LER, RVT, relative yield total (RYT), relative crowding coefficient (RCC) (Table 4), competitive ratio, aggressively, actual yield loss, monetary advantage, and intercropping advantage have been developed to describe competition and economic advantage in intercropping (Ghosh, 2004; Midya *et al*., 2005).

RCC is ability of a species to use limited resource in intercropping with its ability to gain the same resource in intercropping system by using yield comparing and shows the competitive advantage of intercropping components (Snaydon, 1991). RCC of black mustard in most treatment was higher than RCC of pinto been. Its maximum value was observed in treatment non-fertilizer and optimum density of two species about 2.803. The highest value of RCC of pinto been in treatment non-fertilizer and 50% of optimum density of two species. Fertilizer application result in decreasing RCC of pinto bean and increasing

Table 4 - Evaluation of intercropping efficiency of treatme

Fertilizer treatments	Land equivalent ratio (LER)		Relative value total (RVT)		Relative yield total (RYT)		Relative crowding coefficient (RCC) of Pinto bean		Relative crowding coefficient (RCC) of Black mustard	
	Intercropping (50% of optimum density of two species)	Intercropping (optimum density of two species)	Intercropping (50% of optimum density of two species)	Intercropping (optimum density of two species)	Intercropping (50% of optimum density of two species)	Intercropping (optimum density of two species)	Intercropping (50% of optimum density of two species)	Intercropping (optimum density of two species)	Intercropping (50% of optimum density of two species)	Intercropping (optimum density of two species)
Control (non-fertilizer)	1.003	1.102	3.181	4.758	1.003	1.102	1.073	0.356	0.931	2.803
Bio-fertilizers (azotobarvar 1 and barvar 2)	1.107	1.349	2.717	3.874	1.107	1.349	1.007	0.559	0.992	1.787
Bio-fertilizer + 50% chemical fertilizer (urea)	1.253	1.381	2.661	3.229	1.253	1.381	0.879	0.515	1.136	1.940
Bio-fertilizers + 100% chemical fertilizer (urea)	1.304	1.418	2.401	2.817	1.304	1.418	0.804	0.602	1.242	1.658

RCC of black mustard in 50% of optimum density of two species. Also, with increasing black mustard density in intercropping, RCC of black mustard was higher than bean at all fertilizer treatments. Generally, fertilizer application, change the superiority of bean toblack mustard (Table 4).

Fertilizer treatments, particularly bio-fertilizers + 100% chemical fertilizer (urea) improved grain yields of pinto bean and black mustard via higher 100 grains weight and biological yield per unit area. Resource use efficiency was increased in intercropping systems. Intercropping diversify agroecosystem, and resulted in sustainable production and increase economic income, in addition, can be effective the use of agricultural land considerably. Finally, it was concluded that intercropping pattern was better than monocultures of two species at different levels of fertilizers and also bio-fertilizers application could increase efficiency of chemical fertilizer and it can reduce the environmental risk and increase field performance of pinto bean and black mustard.

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Influence of plant biostimulant and spacing on production and postharvest conservation of watermelons cv. Quetzali

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Key words: *Citrullus lanatus*, cold storage, Crop Set®, physico-chemical quality, plant growth regulators.

Abstract: The aim of this study was to evaluate the influence of pre-harvest application of plant biostimulant Crop Set® and different plant spacings on the production attributes and postharvest quality of watermelon 'Quetzali'. The experiment was set up in a completely randomized split-plot (3 × 2 × 4) design, corresponding to three plant spacings (0.40, 0.45 and 0.50 m), application of the plant biostimulant (with and without) and four storage periods at 10°C and RH 90% (0, 14, 21 and 28 days). Fruits were assessed after harvest in terms of average mass of fruits, number of fruits per plant and yield, and throughout the storage periods for flesh firmness, soluble solids content (SSC), titratable acidity (TA), SSC/TA ratio, pH and total soluble sugars (TSS). The average mass of fruit (4.02 kg) was higher in the larger spacing without application of biostimulant. The pre-harvest application of plant biostimulant negatively influenced SSC of fruits, depending on the plant spacing and storage periods. For TA and TSS content, the effect of this product varies with plant spacing and storage days. The lower plant spacing provided higher TSS to the fruits.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai] is a vegetable belonging to the Cucurbitaceae family that has great economic and social expression, with a world production of 118,413,465 tonnes in 2017. Among the four largest producers are China, Turkey, Iran and Brazil, which together are responsible for 76% of global watermelon production (FAOSTAT, 2019).

In Brazil, the Northeast region is an important pole of agricultural crop, with soil and climate conditions favorable for watermelon cultivation throughout the year. Cultural practices are always studied to increase yield and quality of the products. Watermelon is the second most exported vegetable in Brazil and Quetzali is an early commercial cultivar, with

the harvest at 70 days. This cultivar has average mass of 2.5 to 6.0 kg, green skin color with dark and thin streaks, red pulp with few seeds and high soluble solids content, which are desirable characteristics for consumers (Dia *et al.*, 2016).

During the vegetable development an unable cultural management of plant, in the field, can cause irreversible damage in fruit cells, which can affect their shelf life. The management techniques can morphologically and physiologically alter the plant, intervening in its productive potential and affecting the quality and the conservation of the fruits are plant spacing (Gomes *et al*., 2017) and use of plant biostimulants (Martins *et al*., 2013).

Species grown in high densities, especially cucurbits, produce a large number of fruits per area, but with small size, weight and number per plant, which may affect their development, and consequently, the final quality of fruits (Sabo *et al*., 2013; Oga and Umekwe, 2016).

On the other hand, plant biostimulants are substances applied to plants that enhance their nutrition efficiency, abiotic stress tolerance and/or quality traits (Jardin, 2015). They can be defined as mixtures of one or more plant growth regulators with other compounds of a different chemical nature, such as mineral salts (Castro and Pereira, 2008), which are applied in various species of fruits and vegetables with the aim of increasing its production and quality (Leão *et al*., 2005; Costa *et al*., 2008; Martins *et al*., 2013; Aroucha *et al.*, 2018).

Crop Set® (Improcrop-Kentucky-USA) is a commercial product registered in Brazil as foliar fertilizer, containing 1.5% manganese, 1.5% iron and 1% copper, it is a composed of agave (*Yucca shidigera*) extracts and mineral micronutrients with cytokinin-like action (Leão *et al*., 2005). The use of plant regulators belonging to the cytokinin group can increase the fruit size (Tecchio *et al*., 2006; Ainalidou *et al*., 2016) because inducing cell division and thus stimulating cell growth in plant tissues (Taiz *et al*., 2015). Nevertheless, its influence on the yield and watermelon conservation was not reported yet. The watermelon shelf life is around 2-3 weeks at 10-15°C (Maynard, 2001), depending on cultivar. A good quality is reached as soluble solid is above 8% (Tlili *et al*., 2011).

The aim of this study was to evaluate the influence of pre-harvest application of plant biostimulant Crop Set® and different plant spacings on the production attributes and postharvest quality of watermelon cv. Quetzali.

2. Materials and Methods

The experiment was carried out in Mossoró, state of Rio Grande do Norte, Brazil (4° 39' 39" S, 37° 23' 13" W, and 20 m of altitude). The climate of the region according to Köppen climate classification is BSwh type (hot and dry). The region has average annual temperature of 27°C, average annual precipitation of 673.9 mm, unevenly distributed, and air relative humidity of 68.9%.

The soil of the experimental area is classified as Neossolo quartzarenico (Santos *et al*., 2018) and its physical-chemical properties are: pH (H_20) = 5.52; organic matter: 5.5%; P (Mehlich) = 32 mg dm^{-3}; K = 96.5 cmol dm^{-3}; Ca = 1.60 cmol dm^{-3}; Mg = 0.43 cmol dm^{-3}; sand = 935.8 g kg^{-1}; silt = 26.5 g kg^{-1}; clay = 37.7 g kg^{-1}; bulk density = 1.48 g cm^{-3}; soil particle density = 2.69 g dm^{-3}; and total porosity = 0.45 m^3 m^{-3}. The results of the chemical analysis of the irrigation water are: pH = 7.70; electrical conductivity = 2.11 dS m^{-1}; K^+ = 0.12 mmol L^{-1}; Na^+ = 5.02 mmol L^{-1}; Ca^{2+} = 10.43 mmol L^{-1}; Mg^{2+} = 3.05 mmol L^{-1}; Cl^- = 11.48 mmol L^{-1}; CO_3^{-2} = 0.30 mmol L^{-1}; HCO_3^- = 3.70; Sodium adsorption ratio = 1.9 $(mmol^{-1})^{0.5}$; Hardness = 5.4 mg L^{-1}; Cations = 18.7 mmol L^{-1}; and Anions = 15.4 mmol L^{-1}.

The experiment was set up in a completely randomized split-plot (3 × 2 × 4) design, with six replications, each one corresponding to a plant. The plots consisted of plant spacings (2.0 x 0.4 m; 2.0 x 0.45 m and 2.0 x 0.5 m), application of the plant biostimulant Crop Set® (with and without), and postharvest storage (0, 14, 21 and 28 days) in the subplot (Fig. 1).

Seeds of watermelon cultivar Quetzali were used. Plant biostimulant was sprayed with a 20 L backpack sprayer, with stainless steel cone nozzle with flow rate of 615 mL/min, at 18 and 25 days after transplanting, applying 8 and 16 mL of Crop Set®, respectively, regularly on the plants, always in the same way. The dose of the biostimulant was determined according to the manufacturer's recommendations.

The harvest was realized at 65 days after of seedling transplanting. Fruits were transported to Laboratory of Food Technology of the Federal Rural University of the Semi-Arid Region, where part of the fruits were characterized previously by sampling six fruits per treatment. The other part was stored in a cold chamber at 10±2°C and RH 90±1%, for 14, 21 and 28 days. After each storage periods, the fruit quality was evaluated.

Fruits were assessed after harvest for production

in terms of number of fruits per plant, average mass of fruits and yield. At harvest and during cold storage, the quality characteristics were: flesh firmness (N), measured with a 12-mm tip manual penetrometer model 327 FT (McCormick, USA); soluble solids content (SSC, in °Brix), measured with a refractometer (PR - 100, Palette, Atago CO., LTD., Japan); titratable acidity (TA, in % of malic acid), analyzed by titrimetry; SSC/TA ratio; pH, evaluated using a digital potentiometer with glass membrane, calibrated with buffers of pH 7 and 4, according to the method of the Association of Official Analytical Chemists (AOAC, 2016); and total soluble sugars (TSS), measured by the Antrona method, as described by Yemn and Willis (1954), expressing results in percentage (%).

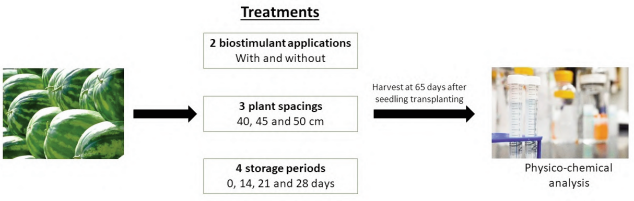

Fig. 1 - Scheme representing the treatments applied to the watermelon fruits.

Data were subjected to analysis of variance and means of the biostimulant and plant spacings factors were compared by the Tukey test ($p \leq 0.05$). The effect of storage periods was evaluated by regression analysis. All statistical analysis were carried out using software Sisvar 5.3 (Ferreira, 2014).

3. Results

There was effect of plant spacing and biostimulant application on the average mass of fruits. While, the production attributes as number of fruits per plant and yield were not affected by plant spacing or application of plant biostimulant (Table 1).

During the fruit storage, there was a significant interaction effect between plant spacing, biostimulant application and storage periods on SSC (Fig. 2), TA (Fig. 3) and TSS (Table 2) of fruits. Also, there was an isolated effect of the storage periods on the SSC/TA

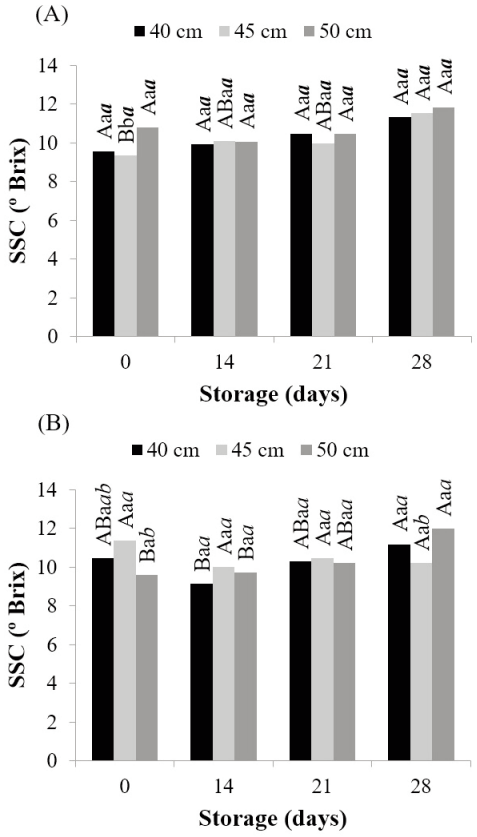

Fig. 2 - Means followed by the same letter do not differ by the Tukey test (p≤0.05). Uppercase letters compare the storage periods within the biostimulant x spacing combination; lowercase letters compare the presence (A) or absence (B) of the Crop Set® application within the storage period x biostimulant combination; italic lowercase letters compare the means of the plant spacings within storage periods x biostimulant combination. DMS for storage periods= 1.811; DMS for biostimulant application= 1.374; DMS for plant spacing= 1.649.

Table 1 - Average mass, number of fruits per plant and yield of 'Quetzali' watermelon depending on Crop Set ® application

Production attribute	Application of crop set	Plant spacing		
		40 cm	45 cm	50 cm
Average mass (kg)	With	3.43 Aa	3.51 Aa	3.92 Aa
	Without	3.41 Ba	3.47 Ba	4.02 Aa
Number of fruits per plant	With	1.16 Aa	1.06 Aa	1.43 Aa
	Without	0.97 Aa	1.39 Aa	1.58 Aa
Yield (t ha^{-1})	With	11.77 Aa	10.20 Aa	13.90 Aa
	Without	10.19 Aa	13.38 Aa	14.29 Aa

Means followed by the same letter do not differ by the Tukey test (p≤0.05). Uppercase letters compare plant spacings and lowercase letters compare application of biostimulant.

ratio, flesh firmness and pH of fruits (Fig. 4).

Fruits without application of biostimulant had the highest average mass in the larger plant spacing (50 cm) (Table 1).

The SSC increased, from 0 to 28 days, in fruit from plants on 45 cm spacing, with Crop Set® application, and non-spayed plants in growth on 40 and 50 cm spacings (Fig. 2). The biostimulant application only influenced on this physicochemical parameter at the day of the harvest, when fruits produced on 45 cm

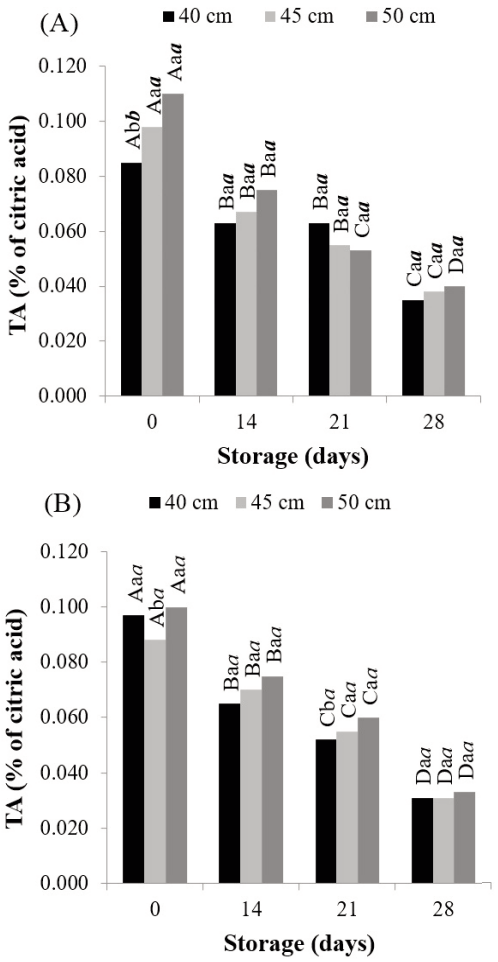

Fig. 3 - Means followed by the same letter do not differ by the Tukey test (p≤0.05). Uppercase letters compare the storage periods within the combination of biostimulant x spacing; lowercase letters compare the presence (A) or absence (B) of the Crop Set® application within the storage period x biostimulant combination; italic lowercase letters compare the means of the plant spacings within storage periods x biostimulant combination. DMS for storage periods= 0.012; DMS for biostimulant application= 0.009; DMS for plant spacing= 0.011.

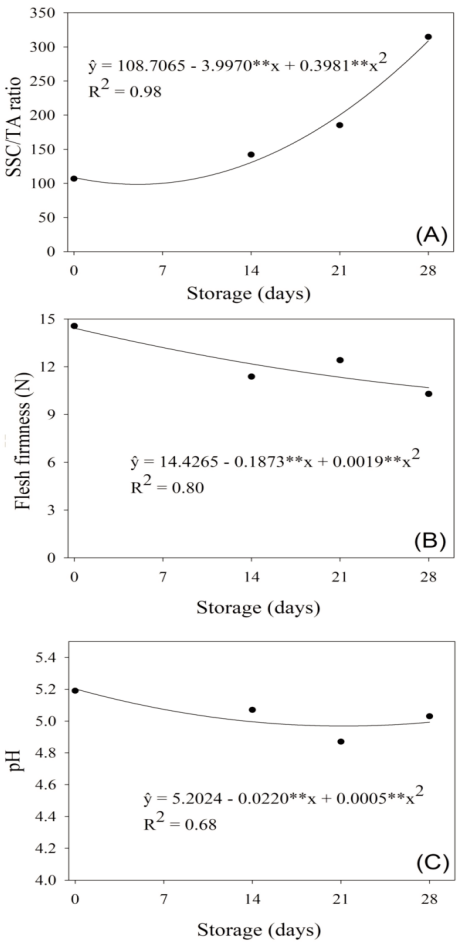

Fig. 4 - SSC/TA ratio (A), flesh firmness (B) and pH (C) of 'Quetzali' watermelon depending on storage periods.

Table 2 - SSC/TA ratio (A), flesh firmness (B) and pH (C) of 'Quetzali' watermelon depending on storage periods

Storage period (days)	Application of Crop Set®			No application of Crop Set®		
	40 cm	45 cm	50 cm	40 cm	45 cm	50 cm
0	7.66 Aa*a*	5.23 Bb*b*	7.80 ABa*a*	6.53 ABa*a*	7.67 Aa*a*	7.66 Aa*a*
14	7.74 Aa*a*	8.30 Aa*a*	8.55 Aa*a*	8.74 Aa*a*	6.91 Aa*ab*	6.26 ABb*b*
21	4.52 Ba*a*	4.80 Ba*a*	4.48 Ca*a*	5.14 Ba*a*	4.36 Ba*a*	4.94 Ba*a*
28	4.18 Ca*a*	4.48 Ba*a*	5.49 BCa*a*	4.43 Ba*a*	5.73 ABa*a*	5.06 Ba*a*

Means followed by the same letter do not differ by the Tukey test (p≤0.05). Uppercase letters compare the storage periods within the combination of biostimulant x spacing; lowercase letters compare the presence (A) or absence (B) of the Crop Set® application within the storage period x biostimulant combination; italic lowercase letters compare the means of the plant spacings within storage periods x biostimulant combination. DMS for storage time= 2.499; DMS for biostimulant application= 1.896; DMS for plant spacing= 2.275.

spacing with Crop Set® application had lower SSC than fruits by plants without application (Fig. 2). The reduction in spacing from 50 to 45 cm possibly increased the competition for water, mineral and luminous resources, leading to a reduction in the size of the fruits and, consequently, to an increase in the SSC due to the concentration effect. Besides that, fruits by non-sprayed plants in 45 and 50 cm spacings showed the highest SSC at 0 and 28 days, respectively. It is important to highlight that fruits produced on 40 cm spacing did not differed from SSC values of higher plant spacing, and therefore is the plant spacing with the best effect on SSC of fruits (Fig. 2).

During the storage, the TA of the fruits decreased as a function of storage periods (Fig. 3). When Crop Set® was applied, there was an effect of plant spacings only at the harvest day, in which fruits of 40 cm spacing shown a lowest TA than fruits of 45 and 50 cm spacings (Fig. 3A). No-application of Crop Set® did not affect the TA of fruits under different spacings (Fig. 3B).

When comparing fruits with and without application, we observed Crop Set® effect at zero and 21 days of storage. At the harvest day, fruits of plants cultivated in 40 cm spacing had lower TA with Crop Set® application, while in 45 cm plant spacing, the fruits had higher TA values with application of plant biostimulant. At 21 days of storage, in fruits by 40 cm plant spacing, Crop Set® application influenced the TA of fruits, increasing this value compared to the fruits without application of the biostimulant (Fig. 3).

With Crop Set application, fruits in 40 cm plant spacing showed a highest sugar content at the first 14 days of storage. On 45 and 50 cm spacing, fruits had the highest sugar content at 14 days, with further reduction of these values (Table 2). In all plant spacings, without Crop Set application, fruits shown high sugar content until 14 days of storage, followed by the decrease of these values. When comparing fruits with and without Crop Set® application, we observed that the plant biostimulant reduced sugar content of 45 cm spacing fruits at harvest. In contrast, this product shown a positive effect on 50 cm plant spacing fruits at 14 days of storage, elevating their sugar content (Table 2).

Comparing the different spatial arrangements in Crop Set® sprayed plants, we observed a lowest sugar content in fruits in 45 cm plant spacing, only at harvest. In plants without application, the 40 cm spacing favored sugar accumulation on the fruits, differing of the highest plant spacing at 14 days of storage (Table 2).

During storage, SSC/TA ratio had an increase of 65% from zero (106.81) to 28 days (314.61) (Fig. 4A). Despite the reduction in both parameters, the more pronounced reduction in TA, compared to SSC, increased the SSC/TA ratio.

The flesh firmness of fruits varied from 14.6 N to 10.9 N during storage, decreasing 25% (Fig. 4B).

During storage, we observed a variation of the pH in the fruits, starting in 5.19 at the harvest and ending in 5.02 at 28 days (Fig. 4C).

4. Discussion and Conclusions

The highest average mass of fruits by non-sprayed plants produced in the larger plant spacing (50 cm) can be attributed to the lower competition among the plants for soil nutrients, water and solar radiation, due to the lower density, which can lead to greater fruit development. Furthermore, a larger plant spacing reduce incidence of diseases, improving the mass of fruits (Bastos *et al*., 2008; Ban *et al*., 2011; Jafari *et al*., 2016).

The use of plant growth regulators can affect crop growth and development, stimulating cell division and increasing nutrient and water uptake (Castro and Vieira, 2001). In this study, the application of the biostimulant may have caused this effect, which reflects the absence of difference between plant spacings in relation to the average mass of fruits by sprayed plants.

Our results showed an increase of SSC in fruits on 45 cm spacing, with Crop Set® application, and non-spayed plants in growth on 40 and 50 cm spacings. According to Yau *et al*. (2010), the SSC of fruits usually decrease after a few days of storage due to the respiration process of the fruit, which is the oxidative breakdown of sugars into simpler molecules. In this case, the increase on the SSC of watermelon fruits observed during storage can be attributed to the solubilization of pectins. The sweetness is the most critical quality trait of watermelon, being mostly influenced by mono- and di-saccharides found in the fruit juice, and partly on other solutes, being all contributes to the juice SSC (Kyriacou *et al*., 2018).

In relation to the Crop Set® application, Martins *et al*. (2013) observed that this biostimulant raised the SSC of 'Quetzali' and 'Style' watermelons. Some studies did not find influence of plant spacing on SSC of watermelon fruits (Bastos *et al*., 2008; Gomes *et al*.,

2017). The SSC in an important quality attribute of watermelon fruits, being desirable that they have values of SSC higher than 8°Brix (Tlili *et al.*, 2011), reached in this study.

Watermelon acidity is mainly attributed to the accumulation of malic acid (Özdemir *et al*., 2016) and its content tends to decrease during storage due to its use as a substrate in respiration (Silveira *et al*., 2013). Corroborating with the results of the present study, Silva *et al*. (2016) and Yau *et al*. (2010) also observed decreasing of the TA of fruits during the watermelon storage. Gomes *et al*. (2017) and Bastos *et al.* (2008) reported in their works that the plant spacing have not influence on TA of watermelon fruits. In its turn, Campagnol *et al*. (2012) found effect of plant spacing on fruit acidity, with the highest values in lowest plant spacing. In relation to Crop Set® application, Martins *et al.* (2013) did not observe an effect of this plant biostimulant on the TA of 'Quetzali' watermelons, differently of 'Style' fruits, which TA had reduction when this product was applied in the plants. Aroucha *et al.* (2018) emphasize that small variations in acidity levels of watermelon fruits are little significant, due to the low concentration of organic acids.

The reduction of sugar content of fruits observed at the last days of storage is related to the respiratory process of the fruits, that involves oxidative degradation of carbohydrates and organic acids.

The SSC/TA ratio of the fruits increased over storage. This parameter in an important indicator of flavor of fruits. Generally, highest SSC/TA ratio indicates a greater sweetness of fruits. This ratio is used to evaluate maturity and palatability of watermelon fruits. Values found in this work are much higher than those pointed by Campagnol *et al*. (2012) in 'Smile' watermelon, which showed SSC/TA ratio variation from 75.55 to 81.88.

The flesh firmness of the fruits decreased 25% over storage. This attribute is important to detect ripeness of watermelon fruits, being associated with the pectin solubilization and depolymerisation (Kyriacou *et al*., 2018). The same way that this work, Martins *et al*. (2013) did not observe effect of Crop Set® application on flesh firmness of watermelons of cultivars Quetzali and Style. Besides that, Campagnol *et al.* (2012) also did not appoint influence of plant spacing on flesh firmness of 'Smile' watermelon fruits.

A small variation on pH was observed on the fruits, and it is explained by the buffer capacity of some fruits, which stabilizes pH even when the decrease of TA is high (Paulson and Stevens, 1974).

In conclusion, the pre-harvest application of plant biostimulant Crop Set® negatively influenced some quality characteristics of 'Quetzali' watermelon, including the decrease of SSC of fruits, depending on the plant spacing and storage periods. In relation to titratable acidity and total sugar content, the effect of this product varies with plant spacing and storage days. The 40 cm plant spacing provided higher total sugar content to the fruits. Still, the 50 cm spacing increased mass of fruit without alter the yield, besides increasing acidity and soluble solids content at the end of storage, being the recommended plant spacing for 'Quetzali' watermelon plants.

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AHS
Advances in Horticultural Science

The first report: The effect of garlic extract on rooting of cuttings of some ornamental plants and fruit trees

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Key words: antimicrobial property, callus, culture substrate, grape, hormone, sycamore, wild privet.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: One of the problems with the use of cuttings, particularly woody and semi-woody ones, is to root them for propagation of various plant species. The use of rooting hormones involves a high cost of foreign exchange and creates many environmental problems. Garlic extract has a lot of antimicrobial properties and can prevent the release of microorganisms in the culture medium of plants. In this study, garlic extract at three concentrations (0, 25, and 50 g/L) was investigated on the rooting of three different species include easy-to-root cuttings (rose, wild privet, and poplar), moderate-root cuttings (sycamore, berry, and grape), and hard-to-root cuttings (apple, sour cherry, and cherry) in three applications of the culture substrate, on the cuttings, and in the callus stage. Results showed that garlic extract can be used at a concentration of 25 g/L at the callus formation stage to improve the quantity and quality of grape cuttings in terms of shooting and rooting. The quantity and quality of shoots and roots for wild privet cuttings can be improved with garlic extract at 25 g/L and be used on the cuttings. Garlic extract at concentrations of 50 and 25 g/L can be used in the callus stage to enhance the quantity and quality of sycamore and berry cuttings, respectively. No positive effects were observed in the other plants, or positive effects were found only either on shooting or on rooting traits.

1. Introduction

Since the uniformity of plants used in gardens and green spaces is important, one of the most effective methods of non-sexual propagation is now the use of various cuttings worldwide. One of the important problems of using cuttings, particularly hardwood and semi-hardwood ones is to root them. To overcome this critical problem, manufacturers apply different methods, one of which is the use of different rooting hormones, particularly auxins (Guan *et al.,* 2015). Although the use of hormones for rooting is very effective, these substances are mostly imported goods that require a large amount of foreign money. In addition, the use of plant-derived substances instead of chemicals and synthetic auxins to deal with adverse environmental effects is an issue that has been considered in the

European Union in recent years. This has led to an increasing interest in the production of substances that can help propagate a variety of plants and, at the same time, are environment friendly (Wojdyła, 2004; Pacholcza *et al.,* 2016). Also, one of the problems during rooting of cuttings is the substrate contamination with pathogens, and consequently the contamination of cuttings and their failure in rooting (Hartmann *et al.,* 2002). According to a previous studies (Wojdyła, 2004; Pacholczak *et al.,* 2016), the use of natural substances can reduce production costs and provide better accessibility compared to chemicals. In addition to various chemical compounds, the use of natural substances in the cultivation of commercial plants is today gaining interest among manufacturers and producers, which has been accompanied by positive and interesting results. In some cases, such substances have even been appropriately replaced chemical compounds due to better results. If plant extracts can be used to root the cuttings, a new step will be taken towards this branch of agricultural science in addition to saving costs and non-dependency on the imports of hormones as well as avoiding environmental contaminants. Plant essential oils play an important role in disinfecting, stimulating, and improving growth, increasing dry matter, and protecting the plant against environmental damages and stresses (Bai *et al.,* 2007).

Garlic extract is a substance with antioxidant, antimicrobial, and antibacterial properties (Harris *et al.,* 2001). Garlic active compounds often accumulate in its underground organ (garlic bulb). Garlic contains sulfide compounds such as allyn, diallyl sulfides, and allicin, with allicin being the most abundant compound in the garlic extract, accounting for approximately 70% of these compounds (Sadaqa *et al.,* 2016). It has been shown that garlic planting in vegetable hydroponic substrates prevents the development of many diseases and reduces microbial population in the substrate (Liu *et al.,* 2014). Garlic extract has many allelopathic chemicals (Wang *et al.,* 2015). The most important chemicals and minerals found in garlic extract are lipids, carbohydrates, fibers, manganese, potassium, sulfur, calcium, phosphorus, magnesium, sodium, vitamin B6, vitamin C, glutamic acid, arginine, aspartic acid, leucine, and lysine (Al Mayahi and Fayadh, 2015).

Given the importance of ornamental trees and flowers and also fruit trees in human health and economics, this study aimed to investigate the possible effects of garlic extract at different concentrations on rooting of cuttings and propagation of rose, wild privet, poplar, sycamore , berry, grape, apple, sour cherry, and cherry cuttings to achieve the best treatment. There is a large body of literature concerning the effect of using hormones on the rooting of cuttings and its optimization (Guan *et al.,* 2015), but no research has so far been conducted on the effect of plant extracts on plant rooting. In the design of this research, therefore, attempts were made to treat the cuttings of different plants with garlic extract in terms of rooting to obtain acceptable results at the beginning of the experiment. This research also tried to use an extract that is suitable for disinfection of the substrate. This was the first report on the use of garlic extract on the rooting of different plants, and it is hoped that the results could pave the ground for further research in this field.

2. Materials and Methods

This research was conducted at the research greenhouse of the Faculty of Agriculture and Natural Resources, University of Arak. To investigate the effect of garlic extract on the rooting of different cuttings, a factorial experiment (cuttings as the first factor and garlic extract concentrations as the second factor) was carried out in a completely randomized design with three replications each with 10 cuttings.

Plant materials

Three types of cuttings including easy-to-root cuttings (rose, wild privet, and poplar), moderate-root cuttings (plane, berries, and grapes), and hard-to-root cuttings (apple, sour cherry, and cherry) were prepared from healthy and genetically uniform native plants in the planting season (December). The cuttings were 10-15 cm in length with a thickness of 5-7 mm.

Extraction of garlic cloves

The garlic cloves were first peeled and grated for each independent experiment. Then, 10 g of the grated garlic was placed in 10 ml of water at room temperature for 24 h. The garlic extract was obtained after passing through filter papers.

Preparation of seedbed, planting of cuttings, and application of treatments

The sandstone culture substrate was similar for all cuttings. Each experiment was consisted of three replicates each with 10 cuttings. Cuttings were grown in 9 rows at 10 cm spacing on a row and 2 cm spacing between the rows in the substrate. The extract con-

centrations were 0 (control), 25, and 50 g/L of distilled water for 2 seconds. The extract was used at three times: 1) extract dispersion on the culture substrate during substrate preparation and before planting the cuttings, 2) at planting the cuttings by inserting the bottom half of the cuttings in the extract to disinfect the cuttings, and 3) dispersion on the substrate at the tips of the cuttings in the callus formation stage. After planting, the cuttings were kept and irrigated regularly under greenhouse conditions for 3 months.

Measuring morphological traits

Immediately after the collection of root and aerial samples, their fresh weight was measured using a digital scale. To determine the dry matter content, the samples were placed in an oven at 70°C for 48 h. Finally, the dry weights of shoots and roots were determined with a digital scale. Further measured traits were the number of roots and shoots, root and shoot length, and longest roots and shoots. During the experiment and the growth of cuttings, the average temperature and relative humidity were 22°C and 35%, respectively, in the greenhouse.

Data were independently normalized with such methods as the removal of outliers (data higher and lower than twice the root mean square error) using the equation X = Y + 0.5, and were then analyzed using SAS 9.4 software. Mean values were compared with Duncan's multiple range test at $p<0.05$.

3. Results

The use of different concentrations of garlic extract in the substrates of cuttings

Shoot-related characteristics. The interaction effect of garlic extract treatment × the substrate and plant type significantly affected the number of green cuttings, total shoot length, number of shoots, mean shoot length, length of the longest shoots, and fresh and dry weights of the shoots. Increasing the extract concentration elevated the number of green cuttings, total shoot length, average shoot length, length of the longest shoots, and fresh and dry weights of shoots in rose cuttings. Elevated number of green cuttings, total shoot length, average shoot length, and length of the longest shoots were observed in wild privet with the extract levels. In poplar cuttings, all the traits improved markedly at a moderate concentration (25 g/L) (Table 1).

The use of garlic extract in sycamore cuttings had no positive effects on shoot-related traits. In berry cuttings, the moderate concentration had positive impacts on the number of green cuttings and total shoot length, but it was not significant on the other traits (Table 1). In grape cuttings, moderate concentration of garlic extract positively affected the number of green cuttings, average shoot length, and shoot fresh and dry weights, while the high concentration (50 g/L) was not significantly different from the moderate one or had a negative effect. In apple cuttings, the moderate concentration significantly increased the number of green cuttings, and both moderate and high concentrations led to increased number of shoots, but the use of garlic extract had no significant effects on the other traits. In sour cherry cuttings, no positive effects were observed on all the traits. In cherry cuttings, the use of garlic extract at both concentrations had slight positive effects on the number of green cuttings, total shoot length, number of shoots, mean shoot length, and length of the longest shoots (Table 1).

In this experiment, the highest number of green cuttings, total shoot length, and the longest shoot length were obtained in wild privet with 50 g/L, with the highest number of shoots and fresh shoots at 25 g/L of garlic extract. The highest number of green cuttings belonging to wild privet cuttings was not significantly different from berry cuttings treated with a moderate concentration. Also, the average shoot length was higher in poplar cuttings treated with a moderate concentration than the other cuttings (Table 1).

Root-related traits. In this experiment, the interaction of garlic extract concentrations × substrate and plant type was significant on total length of roots, root number, mean root length, longest root length, and root fresh and dry weights. Garlic extract at a concentration of 50 g/L had positive effects on total length of roots, root number, mean root length, longest root length, and root fresh and dry weights of rose roots. Total root length and root number were uppermost in wild privet plant at a high concentration (50 g/L). Also, the moderate concentration (25 g/l) produced the longest roots and the highest root fresh and dry weights in this plant (Table 2).

In poplar cuttings, a concentration of 25 g/l resulted in significant increases in total length of roots, root number, mean root length, longest root length, and root fresh and dry weights. There were no significant increases in root traits of sycamore cuttings with the extract application. The extract application was not useful on rooting traits in berry cuttings. Extract

Table 1 - Effects of different concentration of garlic extract (the culture substrate) on shoot-related characteristics

Treatments	Number of green cuttings	Total length of shoots	Number of shoots	Shoot length mean (cm)	The highest shoot length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant							
Rose	0.56 fg	0.89 e	0.67 ef	0.67 ef	0.78 de	0.02 d	0.00 d
Wild privet	7.44 b	181.50 a	33.00 a	5.63 a	19.62 a	5.27 a	0.58 a
Poplar	1.78 de	21.39 d	2.78 e	4.96 b	8.17 b	0.62 c	0.03 c
Sycamore	2.33 d	2.78 e	2.78 e	0.99 e	1.72 d	0.01 d	0.01 cd
Berry	9.11 a	43.04 b	23.71 a	1.78 d	5.44 c	0.05 d	0.01 cd
Grape	7.33 b	33.22 c	11.55 c	2.62 c	8.28 b	2.58 b	0.06 b
Apple	3.89 c	2.89 e	5.78 d	0.50 ef	0.50 de	0.00 d	0.00 d
Sour cherry	1.33 ef	3.89 e	2.22 ef	0.86 e	1.17 de	0.00 d	0.00 d
Cherry	0.22 g	0.11 e	0.22 f	0.11 f	0.11 e	0.00 d	0.00 d
Garlic extract concentration (g·L)							
Control (0 g.L)	3.41 b	23.80 b	7.23 b	1.71 b	4.17 b	0.77 b	0.09 a
25 g·L	4.26 a	34.98 a	9.46 a	2.88 a	6.35 a	1.01 a	0.06 b
50 g·L	3.67 b	20.44 b	7.88 b	1.11 c	4.17 b	0.70 b	0.02 c
Plant × garlic extract concentration (g·L)							
Rose × 0	0.00 j	0.00 g	0.00 i	0.00 j	h00.0	g00.0	f00.0
Rose × 25	0.67 hij	0.67 g	1.00 hi	0.50 ghij	0.50 fgh	g00.0	00.0 f
Rose × 50	1.00 ghij	2.00 g	1.00 hi	1.50 efghi	1,83 fgh	g08.0	ef 01.0
Wild privet × 0	5.00 cd	128.17 c	29.00 bc	5.47 c	17.00 c	b82.5	a72.0
Wild privet × 25	7.67 b	224.75 b	37.50 a	5.37 c	17.50 c	a47.7	b63.0
Wild privet × 50	9.67 a	255.00 a	32.67 b	6.89 b	26.75 a	d00.4	c10.0
Poplar × 0	1.00 ghij	4.67 g	1.00 hi	1.33 efghij	1-33 fgh	g05.0	f00.0
Poplar × 25	3.67 def	59.50 d	4.67 gh	13.55 a	22.67 b	e42.1	cd07.0
Poplar × 50	0.67 hij	0.00 g	2.67 ghi	0.00 j	0.50 fgh	g38.0	ef02.0
Sycamore × 0	2.67 efg	5.17 g	3.00 ghi	1.57 defgh	2.67 fg	g00.0	f00.0
Sycamore × 25	2.33 fgh	3.00 g	2.33 ghi	1.02 fghij	2.00 fgh	g00.0	f00.0
Sycamore × 50	2.00 fghi	0.17 g	3.00 ghi	0.37 hij	0.50 fgh	g05.0	def03.0
Berry × 0	8.33 ab	36.67 ef	19.67 d	1.38 defg	5.67 e	g17.0	ef02.0
Berry × 25	9.67 a	47.47 de	27.00 c	1.67 defgh	5.33 e	g00.0	f00.0
Berry × 50	9.33 ab	45.00 e	26.00 c	1.58 defg	5.33 e	g00.0	f00.0
Grape × 0	7.67 b	27.00 f	11.00 e	2.61 de	7.50 de	f91.0	cde06.0
Grape × 25	8.67 ab	38.33 ef	13.33 e	2.84 d	8.17 d	c49.4	cd08.0
Grape × 50	5.67 c	34.33 ef	10.33 ef	2.42 de	9.17 d	e78.1	cde06.0
Apple × 0	3.67 def	2.33 g	4.67 gh	0.50 ghij	0.50 fgh	0.00 g	0.00 f
Apple × 25	4.33 cde	3.17 g	6.33 fg	0.50 ghij	0.50 fgh	0.00 g	0.00 f
Apple × 50	3.67 def	3.17 g	6.33 fg	0.50 ghij	0.50 fgh	0.00 g	0.00 f
Sour cherry × 0	2.33 fgh	10.17 g	4.00 ghi	2.08 def	2.83 f	0.00 g	0.00 f
Sour cherry × 25	1.00 ghij	1.00 g	3.00 ghi	0.33 hij	0.33 fgh	0.00 g	0.00 f
Sour cherry × 50	67.0 hij	0.50 g	0.67 hi	0.17 ij	0.33 fgh	0.00 g	0.00 f
Cherry × 0	0.00 j	0.00 g	0.00 i	0.00 j	0.00 h	0.00 g	0.00 f
Cherry × 25	0.33 ij	0.17 g	0.33 hi	0.17 ij	0.17 gh	0.00 g	0.00 f
Cherry × 50	0.33 ij	0.17 g	0.33 hi	0.17 ij	0.17 gh	0.00 g	0.00 f
P-value							
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract conc.	0.0072	<0.0001	0.0001	<0.0001	<0.0001	0.0215	<0.0001
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	0.0072	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

Table 2 - Effects of different concentration of garlic extract (the culture substrate) on root-related traits

Treatments	Total length of roots	Number of roots	Root length mean (cm)	The highest root length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant						
Rose	2.61 d	0.55 d	0.36 c	0.33 d	0.2 d	0.00 d
Wild privet	318.75 a	42.57 a	8.43 a	21.22 a	5.27 a	0.58 a
Poplar	29.61 c	3.33 c	4.14 b	7.12 c	0.62 c	0.03 c
Sycamore	0.83 d	0.33 d	0.17 c	0.33 d	0.01 d	0.01 cd
Berry	2.61 d	0.78 d	0.18 c	1.00 d	0.05 d	0.01 cd
Grape	73.06 b	10.12 b	4.84 b	11.00 b	2.58 b	0.06 b
Apple	0.00 d	0.00 g	0.00 c	0.00 d	0.00 d	0.00 d
Sour cherry	0.00 d	0.00 g	0.00 c	0.00 d	0.00 d	0.00 d
Cherry	0.00 d	0.00 g	0.00 c	0.00 d	0.00 d	0.00 d
Garlic extract concentration (g·L)						
Control (0 g·L)	28.67 b	3.44 c	1.92 b	3.48 b	0.77 b	0.09 a
25 g·L	52.83 a	8.27 a	2.43 a	6.00 a	1.01 a	0.06 b
50 g·L	29.74 b	4.64 b	1.41 c	3.48 b	0.70 b	0.02 c
Plant × garlic extract concentration (g·L)						
Rose × 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Rose × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Rose × 50	7.83 g	1.67 f	1.07 f	1.00 e	0.08 g	0.01 ef
Wild privet × 0	202.67 c	21.00 c	9.91 a	19.00 b	5.82 b	0.72 a
Wild privet × 25	418.25 b	56.00 b	8.68 b	28.00 a	7.47 a	0.63 b
Wild privet × 50	462.00 a	a00.67	6.71 c	16.67 b	4.00 d	0.10 c
Poplar × 0	4.33 g	f67.0	0.87 f	1.67 e	0.05 g	0.00 f
Poplar × 25	82.50 e	e33.7	10.18 a	17.33 b	1.42 e	0.07 cd
Poplar × 50	2.00 g	f00.2	0.00 f	0.00 e	0.38 g	0.02 ef
Sycamore × 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Sycamore × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Sycamore × 50	1.00 g	f00.1	0.50 f	1.00 e	0.05 g	0.03 def
Berry × 0	6.50 g	f33.1	25.0 f	2.50 e	0.17 g	0.02 ef
Berry × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Berry × 50	0.00 g	1.00 f	0.31 f	0.50 e	0.00 g	0.00 f
Grape × 0	42.50 f	8.00 e	5.72 cd	9.50 d	0.91 f	0.06 cde
Grape × 25	96.50 d	12.50 d	3.06 e	14.00 c	4.49 c	0.08 cd
Grape × 50	80.17 e	10.67 d	4.75 d	11.00 d	1.78 e	0.05 cde
Apple × 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Apple × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Apple × 50	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Sour cherry × 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Sour cherry × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Sour cherry × 50	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Cherry × 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Cherry × 25	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
Cherry × 50	0.00 g	0.00 f	0.00 f	0.00 e	0.00 g	0.00 f
P-value						
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract conc.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

application in grape cuttings had significant impacts on all rooting traits. Root growing was not observed in the cuttings of apple, sour cherry, and cherry. In this experiment, the highest total root length and number of roots were observed in wild privet cuttings at a high concentration and the highest mean root length was recorded in poplar cuttings at a moderate concentration. The root length and fresh weight were uppermost in wild privet cuttings with a moderate concentration of garlic extract, and the highest root dry weight was measured in the cuttings of this plant in the control treatment (Table 2).

The effects of different concentrations of garlic extract on the cuttings

Shoot-related characteristics. The interaction of plant type and extract concentration resulted in significant impacts on the number of green cuttings, total shoot length, number of shoots, mean shoot length, longest shoot length, and fresh and dry weights of shoots. The application of garlic extract did not have positive effects on all the traits in rose cuttings. In wild privet cuttings, moderate concentration (25 g/L) could further elevate the number of green cuttings, total shoot length, number of shoots, and shoot fresh weight. The longest shoot length and shoot dry weight were observed in wild privet cuttings at high concentration treatment (50 g/L) (Table 3).

The application of garlic extract in poplar cuttings had positive effects on average shoot length and longest shoot length, with no positive impacts on the other traits. Garlic extract at moderate concentration significantly affected all shooting traits in sycamore cuttings. In berry cuttings, the use of this extract at both concentrations led to fairly positive influences on the longest shoot length and fresh and dry weights of the shoots, but no positive effects were observed on the other traits. In grape cuttings, no positive effects were observed on the traits except for the number of green cuttings at 25 g/L and dry weight of shoots at 50 g/L. There was no positive effects on the traits in garlic extract treatments of apple cuttings. Moreover, in sour cherry cuttings, only the mean shoot length was positively influenced by the moderate concentration (Table 3).

In cherry, cuttings treated with a moderate concentration had positive effects on the number of green cuttings, total shoot length, number of shoots, mean shoot length, longest shoot length, and shoot fresh weight. The highest number of green cuttings was found in berry cuttings at all concentrations. Total shoot length and number of shoots were uppermost in wild privet cuttings at moderate-level treatment. In wild privet cuttings, maximum mean shoot length occurred at a concentration of 50 g/l and the longest shoot length was obtained at both moderate and high concentrations. Also, the highest fresh and dry weight of shoots was measured in sycamore cuttings in the control (Table 3).

Root-related traits. Analysis of the experimental data showed that garlic extract yielded significant effects on total length of roots, number of root, mean root length, root length, and root fresh and dry weights of different cuttings. Rose plant cuttings were not rooted in any of the treatments. In wild privet cuttings, an extract concentration of 25 mg/L increased the total root length and both high and moderate concentrations produced the highest roots (Table 4).

Garlic extract treatments had no positive effects on all root traits in poplar cuttings. The application of different extract concentrations in sycamore cuttings led to no significant differences with the control. The berry, apple, sour cherry, and cherry cuttings were not rooted in this experiment. In grape cuttings, the use of garlic extract at both moderate and high concentrations produced positive impacts on the total root length, number of roots, the longest root length, and root fresh and dry weights (Table 4).

Using different concentrations of garlic extract in the callus stage

*Shoot-related characteristics***.** According to the results of ANOVA, the interaction of plant type and garlic extract concentrations led to significant differences in all shoot-related traits at 1% level. The highest number of green cuttings was found in wild privet, berry, and grape cuttings, but there were no significant differences between the control and treatments. A high concentration of garlic extract had a negative effect on the number of green cuttings in poplar, while it had positive effects on rose and sycamore cuttings at both garlic extract concentrations. In apple cuttings, a high concentration of garlic extract positively affected the number of green cuttings. Garlic extract had no effects on sour cherry cuttings and produced negative consequences in cherry cuttings. The highest shoot length was obtained in poplar cuttings treated with 50 g/L of garlic extract. The highest shoots were measured in poplar cuttings treated with garlic extract. There were no significant differences in the shoot length and the longest shoots in wild privet and rose cuttings with garlic extract treatments. The moderate concentration in sycamore cuttings, both concentrations in berry cut-

Table 3 - Effects of different concentration of garlic extract (on the cuttings) on shoot-related characteristics

Treatments	Number of green cuttings	Total length of shoots	Number of shoots	Shoot length mean (cm)	The highest shoot length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant							
Rose	0.33 f	0.50 e	0.44 e	0.50 d	0.44 f	0.15 d	0.01 f
Wild privet	7.78 b	111.00 a	33.25 a	3.46 b	12.78 b	8.34 b	2.23 b
Poplar	6.67 c	71.50 b	9.44 c	8.99 a	18.00 a	11.50 a	2.92 a
Sycamore	1.50 c	3.72 de	2.00 e	1.19 c	1.83 e	1.08 d	0.10 ef
Berry	10.00 a	27.11 c	20.89 b	1.34 c	3.22 d	3.77 c	1.02 d
Grape	6.55 c	30.00 c	9.78 c	3.11 b	7.05 c	8.61 b	1.19 c
Apple	1.78 e	0.69 e	1.78 e	0.28 d	0.33 f	0.16 d	0.03 ef
Sour cherry	2.89 d	6.61 d	4.67 d	1.61 c	3.31 d	0.89 d	0.20 e
Cherry	0.67 ef	0.33 e	0.67 e	0.22 d	0.22 f	0.07 d	0.01 f
Garlic extract concentration (g·L)							
Control (0 g·L)	4.46	29.61 a	8.63	2.31 a	5.04	3.72	0.84 a
25 g·L	4.41	25.37 b	9.78	2.25 a	4.56	3.44	0.64 b
50 g·L	3.96	16.37 c	8.31	1.46 b	5.21	3.15	0.91 a
Plant × garlic extract concentration (g·L)							
Rose × 0	1.00 efg	1.50 g	1.33 i	1.50 gh	1.33 gfh	0.45 f	0.04 h
Rose × 25	0.00 g	0.00 g	0.00 i	0.00 j	0.00 h	0.00 f	0.00 h
Rose × 50	0.00 g	0.00 g	0.00 i	0.00 j	0.00 h	0.00 f	0.00 h
Wild privet × 0	7.33 bc	95.50 bc	23.67 c	3.81 d	11.00 c	6.77 c	1.83 d
Wild privet × 25	8.67 ab	127.83 a	41.33 a	3.08 de	13.33 bc	10.07 b	2.36 c
Wild privet × 50	7.33 bc	104.00 b	35.50 b	3.52 d	14.00 b	8.18 bc	2.49 bc
Poplar × 0	7.67 bc	92.00 c	12.67 d	7.52 c	15.56 b	13.87 a	3.53 a
Poplar × 25	5.67 c	35.50 e	8.00 def	10.10 b	19.00 a	6.93 c	1.53 de
Poplar × 50	6.67 bc	58.75 d	7.67 efg	21.10 a	20.00 a	8.95 b	2.77 b
Sycamore × 0	0.50 efg	0.67 g	2.00 hi	10.1 ghi	1.50 gfh	1.22 ef	0.04 h
Sycamore × 25	2.67 de	7.33 g	3.00 ghi	1.96 fg	3.50 ef	1.08 ef	0.24 h
Sycamore × 50	1.00 efg	0.17 g	1.00 i	0.50ij	0.50 gh	0.23 f	0.03 h
Berry × 0	10.00 a	28.67 ef	20.00 c	1.44 gh	3.00 gf	3.03 de	0.84 g
Berry × 25	10.00 a	30.17 ef	19.00 c	1.70 fgh	3.67 ef	4.31 d	1.01 fg
Berry × 50	10.00 a	22.50 f	23.67 c	0.87 hij	3.00 gf	3.98 d	1.20 f
Grape × 0	6.33 c	33.00 e	8.68 de	3.49 d	6.33 d	6.85 c	1.13 fg
Grape × 25	7.00 bc	29.33 ef	9.39 de	3.40 d	8.38 d	9.17 b	1.08 fg
Grape × 50	6.33 c	34.33 ef	11.33 de	2.42 ef	6.50 d	9.80 b	1.35 ef
Apple × 0	2.33 def	1.17 g	2.33 hi	0.33 ij	0.33 gh	0.10 f	0.01 h
Apple × 25	2.33 def	0.57 g	2.33 hi	0.35 i	0.50 gh	0.37 f	0.08 h
Apple × 50	0.67 efg	0.33 g	0.67 i	0.17 j	17.0 gh	0.02 f	0.01 h
Sour cherry × 0	3.33 d	10.33 g	7.67 efg	1.44 gh	6.00 de	1.14 ef	0.26 h
Sour cherry × 25	2.00 defg	3.67 g	3.67 fghi	1.92 fg	2.00 fgh	0.49 f	0.04 h
Sour cherry × 50	3.33 d	5.83 g	3.67 fghi	1.47 gh	1.25 fgh	1.04 ef	0.32 h
Cherry × 0	0.33 fg	0.17 g	0.33 i	0.17 j	17.0 gh	0.04 f	0.00 h
Cherry × 25	1.33 def	0.67 g	1.33 i	0.33 ij	0.33 gh	0.17 f	0.01 h
Cherry × 50	0.33 fg	0.17 g	0.33 i	0.17 j	17.0 gh	0.01 f	0.01 h
P-value							
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract concentration	0.3226	0.0032	0.2786	0.0819	0.2726	0.8816	0.0007
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

Table 4 - Effects of different concentration of garlic extract (on the cuttings) on root-related traits

Treatments	Total length of roots	Number of roots	Root length mean (cm)	The highest root length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant						
Rose	0.00 d	0.00 c	0.00 c	0.00 d	0.00 d	0.00 d
Wild privet	292.70 a	42.40 a	6.53 b	18.83 b	4.91 a	0.48 a
Poplar	171.61 b	13.87 b	11.83 a	20.87 a	1.95 c	0.16 b
Sycamore	0.33 d	0.22 c	0.15 c	0.22 d	0.03 d	0.00 d
Berry	0.00 d	0.00 c	0.00 c	0.00 d	0.00 d	0.00 d
Grape	98.39 c	13.11 b	7.17 b	17.00 c	3.77 b	0.12 c
Apple	0.00 d	0.00 c	0.00 c	0.00 d	0.00 d	0.00 d
Sour cherry	0.00 d	0.00 c	0.00 c	0.00 d	0.00 d	0.00 d
Cherry	0.00 d	0.00 c	0.00 c	0.00 d	0.00 d	0.00 d
Garlic extract concentration (g·L)						
Control (0 g·L)	46.68 b	4.40 c	3.30 a	5.54 b	1.18 a	0.08 a
25 g·L	63.30 a	7.42 a	2.60 ab	5.00 b	1.25 a	0.06 b
50 g·L	41.18 b	5.60 b	2.28 ab	6.54 a	0.83 b	0.07 b
Plant × garlic extract concentration (g·L)						
Rose × 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Rose × 25	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Rose × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Wild privet× 0	265.50 b	42.00 a	6.33 e	16.00 d	6.99 a	0.57 a
Wild privet× 25	331.50 a	44.33 a	6.25 e	21.00 b	5.34 b	0.45 b
Wild privet× 50	203.50 c	37.00 b	6.92 e	19.50 bc	2.19 cd	0.41 c
Poplar× 0	241.00 b	15.33 c	14.24 a	24.00 a	3.15 c	0.29 d
Poplar × 25	117.67 e	4.50 d	9.82 c	18.00 cd	1.27 d	0.03 g
Poplar × 50	159.17 d	18.67 c	11.23 b	19.67 bc	1.43 d	0.18 e
Sycamore× 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Sycamore × 25	1.00 g	0.00 e	0.44 f	0.67 f	0.00 e	0.00 g
Sycamore × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Berry × 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Berry × 25	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Berry × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Grape × 0	59.50 f	7.33 d	8.33 d	12.00 e	2.45 c	0.03 g
Grape × 25	119.50 e	16.33 c	6.23 e	19.00 bc	4.51 b	0.06 f
Grape × 50	116.17 e	15.67 c	7.34 de	19.67 bc	4.34 b	0.26 d
Apple × 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Apple × 25	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Apple × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Sour cherry × 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Sour cherry × 25	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Sour cherry × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Cherry × 0	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Cherry × 25	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
Cherry × 50	0.00 g	0.00 e	0.00 f	0.00 f	0.00 e	0.00 g
P-value						
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract concentration	0.0658	0.3573	0.0213	0.1200	0.0068	<0.0001
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

tings, and a high concentration in apple cuttings resulted in desirable effects on this trait. The effect of this extract was positive in sour cherry cuttings, and there was a negative effect on the length of shoots produced in cherry cuttings (Table 5).

The fresh weight of shoots was uppermost in wild privet cuttings treated with 25 g/L of garlic extract, which was not significantly different from that of grape cuttings at 50 g/L. Dry weight of wild privet cuttings was the highest in all treatments. The use of garlic extract increased fresh and dry weights of shoots in poplar, sycamore, and berry cuttings. In grape and apple cuttings, a high concentration of garlic extract yielded useful impacts on the fresh and dry weights of shoots while it led to negative consequences in sour cherry and cherry cuttings (Table 5). Overall, the use of garlic extract at the time of callus formation was evaluated to be positive on the shoot-related traits of sycamore, berry, and apple cuttings.

Root-related traits. Analysis of experimental data showed that different concentrations of garlic extract significantly influenced rooting characteristics of different cuttings in the callus stage at 1% level. The use of garlic extract could significantly affect the root number and length at moderate concentration and root dry weight at both concentrations in the callus stage of rose cuttings. The use of moderate concentration had a significant effect on the increase of all root traits in wild privet cuttings in the callus stage. The high concentration caused a decrease in the number of roots and an increase in root length, and the moderate concentration increased the longest root length of poplar cuttings. The use of garlic extract at this stage caused a significant decrease in fresh and dry weights of roots in poplar cuttings. Sycamore cuttings showed some increases in the root traits at the moderate concentration. Berry cuttings increased the number of roots to some extent at both concentrations. In the cuttings of this plant, the length of roots was the greatest at the moderate concentration of garlic extract and the longest root length, and root fresh and dry weights were greater at the concentration of 50 g/L. In grape cuttings, the moderate concentration increased the number of roots and the high concentration elevated root length. In the cuttings of this plant, both concentrations increased the length of the longest root and reduced root fresh and dry weights. Root growing did not occur in apple, sour cherry, and cherry cuttings (Table 6).

The highest number of rooted cuttings was observed in wild privet cuttings treated with the moderate concentration of garlic extract, which did not differ significantly with the grape and control wild privet cuttings at the same concentration. The highest root length was obtained in wild privet cuttings treated with moderate concentration, which did not show significant differences with the cuttings treated with the high concentration. The longest roots and highest root dry weight were recorded in wild privet cuttings at moderate concentration. Poplar control cuttings gained the highest fresh weight (Table 6).

4. Discussion and Conclusions

According to the results of three experimental sections, it seems that the effects of garlic extract on the traits related to shoots and roots of different cuttings depend on the time of use, species type, and the extract concentration. In the first section, the use of high garlic extract concentration (50 g/l) in rose cuttings and moderate concentration (25 g/l) in wild privet, poplar, berry, grape, apple, and cherry cuttings had positive impacts. Garlic extract had no positive effects on shoot traits in sycamore and sour cherry culture substrates. In *Schefflera arboricola*, garlic extract application increased plant height, leaf number, and fresh and dry weights of leaves (Hanafy *et al.,* 2012). In leguminous plants, the extract especially at high concentrations caused negative effects on growth and decreased plant height (Adeleke, 2015). Also, using a high concentration of garlic extract in the culture substrates of rose and wild privet, and moderate concentration in poplar and grape substrates had positive effects on their rooting traits. However, a high concentration of garlic extract was not effective in the rooting of sycamore, berry, apple, sour cherry, and cherry cuttings. Considering the evaluated shoot and root traits, the use of 50 g/L and 25 g/L of garlic extract was useful in rose cuttings and poplar and grape cuttings, respectively.

Garlic extract contains many allelopathic chemicals (Wang *et al.,* 2015) with a known antimicrobial and antibacterial agent (Harris *et al.,* 2001). Garlic planting in vegetable hydroponic substrates has been shown to prevent them from many diseases and to reduce the microbial population in the substrate (Liu *et al.,* 2014). The positive effects of garlic extract are more evident at low and moderate concentrations. High concentrations of garlic extract led to decreased growth of shoots and roots due to increased allelo-

Table 5 - Effects of different concentration of garlic extract (in the callus stage) on shoot-related characteristics

Treatments	Number of green cuttings	Shoot length mean (cm)	The highest shoot length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant					
Rose	1.22 cd	5.46 c	10.44 d	2.27 d	0.70 e
Wild privet	9.67 a	8.54 b	29.78 b	23.08 a	7.09 a
Poplar	4.89 b	18.14 a	35.14 a	13.08 b	4.76 b
Sycamore	0.78 d	2.14 de	2.44 e	0.40 de	0.08 ef
Berry	8.78 a	3.66 cd	10.33 d	5.26 c	1.43 d
Grape	8.67 a	8.41 b	15.39 c	21.81 a	2.98 c
Apple	1.22 cd	1.45 e	1.61 e	0.17 e	0.03 ef
Sour cherry	2.33 c	2.46 de	3.83 e	0.53 de	0.09 ef
Cherry	0.33 d	0.78 e	0.50 e	0.03 e	0.01 f
Garlic extract concentration (g·L)					
Control (0 g·L)	4.00	5.08	9.94 b	6.47 b	1.65 b
25 g·L	4.59	5.36	12.50 a	7.82 a	2.08 a
50 g·L	4.04	6.10	12.27 a	6.06 b	1.88 a
Plant × garlic extract concentration (g·L)					
Rose × 0	0.67 def	5.73 cdef	11.67 cdef	1.84 fgh	0.33 hi
Rose × 25	2.00 cdef	4.98 defg	9.00 efgh	3.19 efgh	0.92 ghi
Rose × 50	1.00 cdef	5.66 def	10.67 defg	1.79 fgh	0.84 ghi
Wild privet × 0	9.67 a	8.54 cd	30.17 b	21.34 b	6.95 a
Wild privet × 25	10.00 a	8.81 c	27.83 b	26.27 a	7.41 a
Wild privet × 50	9.33 a	8.26 cd	31.33 b	20.89 b	6.92 a
Poplar × 0	5.33 b	17.27 b	26.50 b	10.19 d	3.78 cd
Poplar × 25	6.33 b	15.03 b	37.67 a	14.92 c	5.51 b
Poplar × 50	3.00 c	21.09 a	40.00 a	13.79 c	4.66 bc
Sycamore × 0	0.00 f	0.00 i	0.00 j	0.00 h	0.00 i
Sycamore × 25	1.33 cdef	5.00 defg	5.67 ghij	0.48 gh	0.15 hi
Sycamore × 50	1.00 cdef	1.43 ghi	1.67 ji	0.73 gh	0.00 i
Berry × 0	8.33 a	3.08 efghi	5.00 fghi	3.03 de	1.14 ghi
Berry × 25	9.00 a	4.08 efgh	11.83 cdef	5.10 ef	1.35 fgh
Berry × 50	9.00 a	3.82 fghi	12.17 cdef	6.66 e	1.81 fg
Grape × 0	8.67 a	8.45 cd	17.00 c	21.71 b	3.31 de
Grape × 25	9.00 a	7.66 cde	13.67 cde	21.88 b	3.18 de
Grape × 50	8.33 a	9.13 c	15.50 cd	25.87 a	2.46 ef
Apple × 0	0.67 def	1.00 hi	0.83 ji	0.03 h	0.03 i
Apple × 25	0.67 def	1.00 hi	0.83 ji	0.06 h	0.01 i
Apple × 50	2.33 cde	3.42 fghi	3.17 hij	0.41 gh	0.06 i
Sour cherry × 0	2.00 cdef	0.67 hi	1.00 ji	0.28 gh	0.06 i
Sour cherry × 25	2.67 cd	3.54 fghi	5.33 ghij	0.49 f	0.17 hi
Sour cherry × 50	2.33 cde	3.17 fghi	5.17 ghij	0.48 gh	0.05 i
Cherry × 0	0.67 def	1.00 hi	0.83 ji	0.07 h	0.01 i
Cherry × 25	0.33 ef	1.33 ghi	0.67 j	0.03 h	0.01 i
Cherry × 50	0.00 f	0.00 i	0.00 j	0.00 h	0.01 i
P-value					
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract concentration	0.1060	0.1614	0.1060	0.0159	0.1672
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

Table 6 - Effects of different concentration of garlic extract (in the callus stage) on root-related traits

Treatments	Number of roots	Root length mean (cm)	The highest root length (cm)	Fresh weight (gr)	Dry weight (gr)
Plant					
Rose	1.22 d	5.60 c	7.83 c	0.40 d	0.04 d
Wild privet	9.00 a	16.59 a	32.79 a	13.04 b	4.40 a
Poplar	3.87 c	14.91 a	24.74 b	6.31 c	0.80 c
Sycamore	0.44 ef	1.28 d	2.28 d	0.04 d	0.01 d
Berry	1.00 de	8.82 b	8.07 c	0.35 d	0.06 d
Grape	b14.8	10.09 b	26.78 b	14.65 a	2.59 b
Apple	0.00 f	0.00 d	0.00 d	0.00 d	0.00 d
Sour cherry	0.00 f	0.00 d	0.00 d	0.00 d	0.00 d
Cherry	0.00 f	0.00 d	0.00 d	0.00 d	0.00 d
Garlic extract concentration (g·L)					
Control (0 g·L)	2.08 b	4.22 b	7.19 b	4.45 a	0.64 b
25 g·L	3.08 a	7.09 a	12.86 a	3.57 b	0.87 a
50 g·L	2.26 b	7.01 a	12.35 a	2.27 c	0.64 b
Plant × garlic extract concentration (g·L)					
Rose × 0	0.67 fg	4.78 g	7.33 e	0.25 e	0.00 g
Rose × 25	2.33 e	6.62 fg	8.33 e	0.30 e	0.03 f
Rose × 50	0.67 fg	5.39 g	7.83 e	0.65 e	0.09 f
Wild privet× 0	9.00 ab	14.33 bc	26.50 bc	12.89 bc	3.63 b
Wild privet× 25	10.00 a	19.26 a	35.67 a	13.96 b	5.06 a
Wild privet× 50	8.00 bc	16.31 ab	32.00 ab	11.89 bc	3.80 b
Poplar× 0	5.00 d	11.24 cde	17.07 d	17.24 a	1.18 d
Poplar × 25	4.50 d	14.80 b	30.67 ab	3.05 d	0.50 ef
Poplar × 50	2.33 e	18.68 a	26.50 bc	2.29 de	0.73 de
Sycamore× 0	0.00 g	0.00 f	0.00 f	0.00 e	0.00 f
Sycamore × 25	1.00 fg	3.42 gh	3.83 ef	0.09 e	0.01 f
Sycamore × 50	0.33 fg	0.00 h	3.00 ef	0.02 e	0.00 g
Berry × 0	0.33 fg	0.00 h	0.00 f	0.03 e	0.03 f
Berry × 25	1.33 ef	13.90 bcd	7.50 e	0.24 e	0.03 f
Berry × 50	1.33 ef	9.62 ef	13.83 d	0.78 e	0.13 f
Grape × 0	7.00 c	9.64 ef	24.33 c	18.68 a	3.99 b
Grape × 25	9.00 ab	9.84 ef	28.00 bc	14.47 b	2.18 c
Grape × 50	7.67 c	10.80 de	28.00 bc	10.89 c	2.06 c
Apple × 0	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Apple × 25	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Apple × 50	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Sour cherry × 0	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Sour cherry × 25	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Sour cherry × 50	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Cherry × 0	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Cherry × 25	0.00 g	0.00 h	0.00 f	0.00 e	0.00 f
Cherry × 50	0.00 g	h00.0	0.00 f	0.00 e	0.00 f
P-value					
Plant	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Garlic extract concentration	<0.0001	<0.0001	<0.0001	<0.0001	0.0474
Plant × garlic extract concentration (g·L)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Mean values followed by the similar letters within a column are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

pathic properties (Adeleke, 2015). One of the reasons for the failure of rooting in the cuttings is the substrate contamination to pathogenic agents and their entry into the stem, and as a result, the contamination of cuttings and failure in rooting (Hartmann *et al.,* 2002). Garlic extract is a strong disinfectant that resulted in increased rooting and improved shooting.

In the second part of the experiment, using the moderate concentration of garlic extract had positive impacts on the shoot and root traits of wild privet cuttings. Garlic extract contains auxin hormone, and immersing the tips of olive cuttings in garlic extract in combination with other natural auxins-containing substances (e.g., algae) produced the longest roots and increased the number of shoots, leaf number, mean shoot length, and dry weight of shoots (Gad and Ibrahim, 2018). The same authors reported that the compound was able to compete with IBA and no significant differences were found in these traits with those of IBA-treated olive cuttings.

In the third part of the experiment, the use of moderate-level garlic extract in the callus stage of rose and sycamore cuttings and the high concentration in the callus stage of the berry cuttings had positive effects on the shoot and root traits. There is ample evidence that garlic extract has an allelochemical property that affects cell division, absorption of water and minerals, phytohormone metabolism, respiration and photosynthesis, enzyme function, and expression of genes (Portales-Reyes *et al.,* 2015; Sadaqa *et al.,* 2016).

Biostimulators are a group of naturally occurring substances that can be applied either as spraying or at the tips of cuttings, and some of these substances increase water absorption and nutrient transfer, and stimulate growth processes, with possible hormonal properties (Dobranszki and Teixeira da Silva, 2010); a few of them had positive effects on ornamental pine propagation and rooting of ornamental plant cuttings (Shevchenko, 2008; Szabó and Hrotkó, 2009; Pacholczak *et al.,* 2016). These substances stimulate the processes occurring in plants to increase growth. Khan *et al*. (2009) and Borowski (2009) reported that natural substances alter the growth and development of cells in the root system and the concentration of many substances in the plant. The mechanism of the physiological and biochemical processes of these substances remains unknown, and their information and application in horticulture and plant propagation are very limited and require further study and attention (Du Jardin, 2015; Ertani *et al.,* 2015).

Many plant extracts such as coconut juice, banana pulp, potato puree, date sap, corn extract, papaya extract, and beef extract have been used in micropropagation to enhance plant growth (Islam *et al.,* 2003; Murdad *et al.,* 2010; Nambiar *et al.,* 2012; Sudipta *et al.,* 2013). The accumulation of carbohydrates, particularly simple ones (monomers), in the rooting zone is essential for rooting onset at early stages, and carbohydrate concentrations of applied natural substances had high influences on the rooting (Costa *et al.,* 2007).

Active oxygen species (ROSs) are oxygen species that destruct cells and destroy membranes and nucleic acids during such tensions as cutting and mechanical damage to cuttings. ROSs, therefore, is always present at rooting event. Biostimulators and natural substances used to increase the growth of different plants reduce the effects of various stresses on plants (Dobranszki and Teixeira da Silva, 2010). Phenolic compounds are classified into simple phenols, phenolic acids, hydroxycinnamic derivatives, and flavonoids. Researchers have reported that many phenolic compounds and flavonoids function as strong antioxidant compounds that are found abundantly in many plant extracts, including medicinal plants. The active ingredients of medicinal plants include complex chemical compounds produced and stored in the organs of medicinal plants and protect cells from oxidative damage (Lin and Harnly, 2010). The extracts of medicinal plants have many antimicrobial properties and can prevent the release of microorganisms in the culture medium and plant growth media (Radwan *et al.,* 2015).

Considering the shoot and root traits evaluated in the three experiments, the use of 50 g/L and 25 g/L of garlic extract was useful in rose cuttings and poplar and grape cuttings, respectively. Moderate concentration (25 g/L) of garlic extract revealed positive effects on shoot and root traits of wild privet cuttings. Also, the use of moderate garlic extract had positive effects on rose, and at a high concentration (50 g/L) on sycamore cuttings and on berry cuttings in the callus stage. As a result, to improve the quantity and quality of shooting and rooting in rose cuttings, garlic extract can be used at a concentration of 50 g/L in the substrate or 25 50 g/L in the callus formation stage. A comparison of data from these two experiments indicated that the use of 25 g/L was more effective in the callous formation stage. Also, to improve the quality and quantity of shoots and roots in wild privet cuttings, garlic extract can be used at 25 g/L on the cuttings. The quality and quantity of

poplar and berry cuttings can be improved by concentrations of 50 g/L and 25 g/L of garlic extract in the callus stage. No positive impacts were noticed on the other plants, or positive effects were seen only on shooting or rooting traits.

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Aloe vera coatings maintain antioxidants of fig (*Ficus carica* L.) fruit during storage

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Abstract: Demand for fig fruit is increasing because of its high antioxidant capacity and health benefit for human. So, interest in maintaining bioactive components of fruit has been increased. However, antioxidant capacity of fruits decreases during storage. Fresh fruits of the well-known fig (*Ficus carica* L.) cultivar 'Siyah' were grown in Fars Province, Iran, treated with different concentrations of Aloe vera gel prior to being placed into cold storage. We examined fruit for firmness, soluble solids contents, anthocyanin concentrations, total phenolic compounds, flavonoid concentrations, ascorbic acid content, antioxidant activity, phenylalanine ammonia-lyase and superoxide dismutase activities. Figs coated with *Aloe vera* gel maintained higher firmness, anthocyanin concentrations, total phenolic compounds, flavonoid concentrations, ascorbic acid content, and antioxidant capacity than the control. Phenylalanine ammonia-lyase and superoxide dismutase activities were enhanced with Aloe vera gel treatments. The results showed that Aloe vera gel treatments could be an alternative to the current chemical protocols for preserving nutraceutical traits of fig fruit by maintaining antioxidant capacity during storage.

1. Introduction

Fig fruit is an important part of the Mediterranean food and is rich in fibers and antioxidant compounds (Arvaniti *et al.,* 2019). Antioxidant compounds are rich in fruits like fig and can prevent free radical formation. Foods which are rich in phytochemicals decrease incidence of disease and maintain body healthful (Singh, 2016). For this reason, it is important to use these phytochemicals in our diet (Singh, 2016). It has been detected that anthocyanin intake can prevent heart diseases and promote anti-carcinogenic and hypoglycemic activities. Thus, high content of anthocyanin has made fig attractive for consumers. Also, figs con-

tain high level of flavonoid which reduces oxidative stress (Reyes-Avalos *et al.,* 2016). Delicate epidermal tissue, climacteric behavior and extensive softening make fig fruit susceptible to the wounding and spoilage which limit shelf life of fruit (Bahar and Lichter, 2018). Also, it has been confirmed that antioxidant activity of fruits decreases during storage (Madani *et al.,* 2016). Therefore, maintenance of antioxidants of fig fruit during storage is important, because of their health benefits for human.

Several strategies have been implemented to maintain bioactive compounds of fruit during storage. Among them, coatings are popular because of their biodegradable and non-toxic materials (Reyes-Avalos *et al.,* 2016; Allegra *et al.*, 2017). They provide a barrier against gas transfer, which delays ripening and improves taste and texture (Khaliq *et al.,* 2019). The Aloe vera gel compounds are mainly polysaccharides, minerals, sugars, vitamins, and antioxidant agents like phenolic compounds (Rasouli *et al.,* 2019). Application of Aloe vera gel has received increased attention by the food industry due to its effectiveness for increasing shelf-life of fresh products and increasing antioxidant activity (Sogvar *et al.,* 2016). Moreover, Aloe vera gel maintained antioxidant activity of button mushrooms (Mirshekari *et al.,* 2019) and sapota fruit (Khaliq *et al.,* 2019). However, few studies have addressed the effects of edible coatings on quality of fig under cold storage conditions. Application of alginate-chitosan decreased fungal contamination and increased firmness of fig fruit under cold storage (Reyes-Avalos *et al.,* 2016).

Thus, the objective of this study was to determine the effects of Aloe vera gel application at different concentrations for maintaining phytochemicals of fig fruit during cold storage. We examined several beneficial phytochemicals (anthocyanin, total phenolic compound, total flavonoid concentration, and ascorbic acid), antioxidant activity, and phenylalanine ammonia-lyase and superoxide dismutase activities.

2. Materials and Methods

Plant materials and treatments

Mature harvested figs (*Ficus carica* L.), cv. Siyah (black, firm with 13% soluble solids content) grown in Fars Province, Iran, were transported to the Faculty of Agriculture of Yasouj University. Mature leaves of greenhouse-grown Aloe vera plants were excised. The leaf matrix was detached from the outer cortex, and then the hydroparenchyma was mixed in a blender. The mixture was filtered through cheesecloth to remove the fibers, and the filtrate constituted fresh Aloe vera gel (Sogvar *et al.,* 2016). Defect-free fruits with uniform size and color were divided into four groups, each group received one of four treatments: 1) control (0%), 2) Aloe vera gel 1:3 (25%), 3) 1:1 (50%), and 4) 3:1 (75%). After that, all fruits were placed into plastic trays (285 x 125 x 65 mm) over-wrapped with plastic films [0.02 mm-thick polyvinyl chloride (PVC)]. Trays with fruit were stored at 2±1°C and 85-90% RH for 15 days (cold storage). Analyses were performed after 0, 3, 6, 9, 12, and 15 days of cold storage.

Firmness, soluble solids (SSC) and ascorbic acid content

A digital fruit hardness tester (STEP Systems GmbH, Germany) with a 5-mm diameter probe was used to determine fruit firmness (expressed in Newtons (N). Five g of the homogenate of a composite sample using a kitchen blender (Nu-777, Nautiunl, Japan) with 40 mL of distilled water was used for analyses of chemical parameters (Ranganna, 1986). Then samples filtered through cotton wool. SSC was measured with a handheld refractometer (Atago-Pal1, Tokyo, Japan) and expressed as percent. Ascorbic acid content was determined using the 2, 6-dichlorophenolindophenol dye titration method described by Mirshekari *et al.* (2017). Five g of fruit tissues homogenized in 90 ml of 3.0% metaphosphoric acid solution. An aliquot of the sample (10 ml) was titrated against 2, 6-dichlorophenolindophenol dye until a pink color persisted for 15 s and results expressed as mg ascorbic acid per 100 g of fresh weight (FW).

Anthocyanin and flavonoid concentration (FC)

Anthocyanin content in fruit samples was measured by the pH differential method described by Hassanpour (2015). Two g of samples was added to the 20 ml of methanol containing HCl (1%). The mixture was centrifuged at 17,000 g for 15 min at 4 °C. Absorbance of supernatant was measured in a spectrophotometer (Shimadzu, USA) at 530 and 700 nm in buffers at pH 1.0 and 4.5, using following formula:

$$A = [(A_{530}-A_{700})\ pH_{1.0} - (A_{530}-A_{700})\ pH_{4.5}]$$

Results were expressed as mg of cyanidin-3-O-glucoside equivalents per 100 g of FW.

FC was measured according to the method described by Saba and Sogvar (2016). Extraction from four g of the sample was done using 50 ml methanol. One mL aliquot of catechin standard solution (0-100

mg L^{-1}) or samples were added to 10 mL volumetric flasks containing 4 mL water. Initially 0.3 mL of 5% $NaNO_2$ was added to the flask, following 0.3 mL of 10% $AlCl_3$ was added after 5 min, and then 2 mL of 1 M NaOH was added to the mixture. Immediately, the solution was diluted to a final volume of 10 mL with water and mixed thoroughly. The absorbance was measured at 510 nm, using a spectrophotometer (UV/Vis Perkin Elmer, Lambda EZ201, USA) and result was expressed as mg catechin equivalents per 100 g of FW.

Total phenolic compounds (TPC) and antioxidant activity

Fruit extract was prepared using the method described by Ong *et al.* (2013). Fruit tissue (2 g) was homogenized in a glass tube with 10mL of methanol (80%). The mixture was then incubated at 45°C for 1 h. For TPC measurement 0.1 mL of the crude extract solution was placed in a test tube and 0.1 mL distilled water in a test tube served as the control (blank). Then six millilitres of water was added to the sample and blank. After that, 0.5 mL undiluted Folin-Ciocalteu reagent was added to the mixtures. Between 30 s and 8 min later, 1.5 mL saturated sodium carbonate was added. Then 1.9 mL water was added to the solutions to give a final volume of 10 mL and the mixture vortexes and incubated for 2 h at 35°C. The absorption of TPC was determined at 765 nm using a spectrophotometer. A calibration standard curve was established using gallic acid. TPC was determined against the standard gallic acid calibration curve and the absorbance value was converted to mg of gallic acid equivalents (GAE) per 100 gram of fresh weight (mg GAE 100 g^{-1} FW) (Ong *et al.,* 2013).

Antioxidant capacity (ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) assays were determined spectrophotometrically, according to Benzie and Strain (1996) and Kerem *et al.* (2006), respectively. The results were expressed as Fe^{2+} equivalents mM kg^{-1} for FRAP and µM trolox equivalents for TEAC in 100 g of FW.

Phenylalanine ammonia-lyase (PAL) and superoxide dismutase (SOD) activities

Fig samples were frozen in liquid nitrogen and then stored at -80°C until analysis. Each frozen sample (10 g) was ground with a mortar and pestle and used to determine PAL and SOD activity. Two g of samples was homogenized in a 4 mL solution containing 0.05 mol L^{-1} Tris-HCl buffer (pH =7.5), 3 mmol L^{-1} $MgCl_2$ and 1 mmol L^{-1} EDTA at 4°C. The homogenate was then centrifuged at 25,000 g for 20 min at 4°C and the supernatant was used as the crude extract for SOD and PAL assays (Maghoumi *et al.,* 2013). Measurement of PAL activity was performed at 290 nm, according to the method described by Aghdam *et al.* (2012). PAL activity was evaluated as nM cinnamic acid h^{-1} mg^{-1} protein. The procedure for assay of SOD was performed using methods described by (Maghoumi *et al.,* 2013). SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium. The absorbance by the reaction mixture was read at 560 nm. Concentration of protein of the extracts was determined according to Bradford (1976) with bovine serum albumin as a standard. Enzyme activity was expressed as unit mg^{-1} protein.

Sensory evaluation

Texture, taste and overall quality of samples analyzed by ten trained panelist after 15 days at cold storage (2±1°C) and transferring fruits to the room temperature for sensory analysis. The sensory were evaluated using a hedonic scale 1-5, where 1= very poor, 2= poor, 3= fair, 4= good and 5= excellent.

Experimental design and statistical analysis

All experiments were conducted within a completely randomized design (CRD). Data were pooled before analysis and the whole experiment was repeated three times. In each biological and technical experiment 328 fruit were used. There were four replicates per treatment in each experiment. Data were subjected to analysis of variance using the Statistical Analysis System (SAS, ANOVA procedure) version 8.2 (SAS Institute Inc., Cary, NC, USA). The means were compared with the Duncan's Multiple Range Test (DMRT) at significance level of 0.05.

3. Results and Discussion

Firmness, SSC and ascorbic acid content

Firmness is the key factor of quality and which changes during ripening (Madani *et al.,* 2014). Fruit firmness was reduced during storage irrespective of treatment, but Aloe vera gel treated fruits softened more slowly (Fig. 1A). Short postharvest life of fig fruit is because of softening and epidermal cracking (Villalobos *et al.,* 2016). Aloe vera film acts as a barrier which prevents O_2 uptake on fruit, thereby decreases softening and ripening processes (Hassanpour, 2015). Moreover, polygalacturonase and pectin methylesterase activity increases during ripening, and this causes fruit softening (Madani *et*

al., 2014). The results are comparable with Reyes-Avalos *et al.* (2016) who indicated that alginate-chitosan coating could maintain firmness of fig fruit during storage. Aloe vera gel might decrease polygalacturonase and pectin methylesterase activity and thereby maintain firmness of fig fruit during cold storage.

There were significant differences in the SSC among treatments. SSC of control fruits increased during storage (Fig. 1B). However, Aloe vera gel treated fruits had the lowest SSC compared to the control during cold storage. The increase in SSC might be related to the solubilization of polyuronides and hemicelluloses of fruit cell walls and hydrolysis of insoluble polysaccharide into simple sugars (Tanada-Palmu and Grosso, 2005). These results are comparable with Rasouli *et al.* (2019) and (Martínez-Romero *et al.,* 2017) who mentioned that Aloe vera gel reduced the SSC of orange and plum fruit, respectively. Aloe vera gel might decrease respiration rate and SSC in fig fruit.

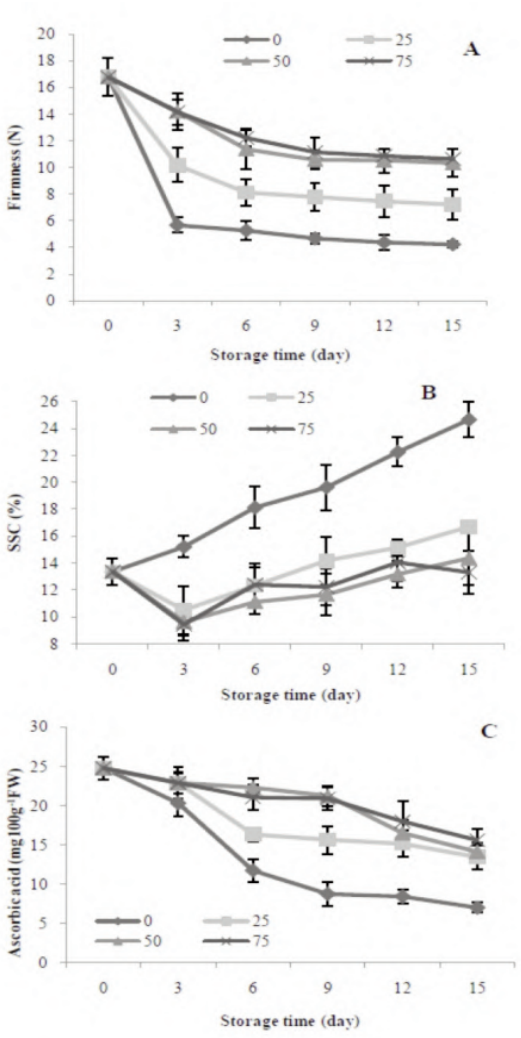

Fig. 1 - Firmness (A), soluble solid concentration (SSC) (B) and ascorbic acid content (C) values in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%). Fruit were stored at 2°C. Vertical bars represent standard error of means of three experiments with four replicates per experiment.

During cold storage, ascorbic acid content of control fruit decreased from 24.75 mg per 100 g of FW to 7 mg per 100 g of FW (Fig. 1C). However, Aloe vera gel treated fruits maintained ascorbic acid content relative to the control. Ascorbic acid is the most important antioxidant which decreases the damage of ROS (Rasouli *et al.,* 2019). Autoxidation causes ascorbic acid losses during storage when combines with oxygen in the air (Baraiya *et al.,* 2015). Aloe vera gel might causes a barrier layer for gas and decreases oxidation of ascorbic acid which caused by ascorbate oxidase enzyme in the presence of oxygen (Sogvar *et al.,* 2016). Since ascorbic acid has beneficial effect on human health, the positive effects of Aloe vera gel on maintaining ascorbic acid content of fig fruit can be interested for nutraceutical purposes.

Anthocyanin, FC, TPC and antioxidant capacity

Fig fruit is rich in phenolic compounds, which are responsible for antioxidant activity (Ercisli *et al.,* 2012). Anthocyanin of non-treated fruits at harvest and after 15 days of cold storage was 11.12 and 21.45 of mg cyanidin-3-O-glucoside equivalents per 100 g FW, respectively. (Fig. 2A). Aloe vera gel 50% and 75% treatments increased anthocyanin after 15 days at cold storage relative to the control. (Fig. 2A). Moreover, FC of Aloe vera gel treatments was significantly higher than control during cold storage (Fig. 2B). TPC decreased during cold storage regardless of treatments; but TPC of treated fruits were significantly higher than that of non-treated fruits (Fig. 2C). From a biological and nutritional perspective, the antioxidant capacity of anthocyanin is important (Wang *et al.,* 1996); therefore, maintaining anthocyanin is potentially beneficial. It has been also reported that strawberries, and bush blueberry treated with chitosan maintained higher levels of anthocyanin (Wang and Gao, 2013; Chiabrando and Giacalone, 2015).

Flavonoids are water soluble polyphenolic molecules which have health promoting effects like antioxidants, radical scavengers, anti-mutagenic, anti-inflammatory, anti-carcinogen, and anti-depressant (Singh, 2016). Moreover, with their antioxidant activity, they increase shelf life of fruits and vegetables (Ververidis *et al.,* 2007). Nair *et al.* (2018) showed higher FC in guava fruit treated with chitosan coatings. Also, (Khaliq *et al.,* 2019) indicated that

Aloe vera gel treated sapodilla fruits had higher FC.

Phenols are one of the most important antioxidant compounds of fruits and vegetables. The application of coatings like Aloe vera gel might delay senescence and decrease TPC loss during storage (Rasouli *et al.,* 2019). Increase in anthocyanin, FC and TPC of fruit treated with Aloe vera gel is comparable to those reported previously by Hassanpour (2015) for raspberry fruit treated with aloe vera gel. This may be related to the persistent biosynthesis of anthocyanin, flavonoids and TPC after harvesting. Also, enzymes which are involved in biosynthesis process of TPC, flavonoids and anthocyanin like PAL might be up regulated with Aloe vera gel treatment. The higher nutraceutical compounds detected in loquat fruits coated with chitosan could be related to the lower ROS due to the long-time physiological stress of storage (Petriccione *et al.,* 2015). Therefore, Aloe vera treated fruit might have a protective effect on nutraceutical compounds in delaying their oxidative processes and bio-transformation during storage.

Figs treated with Aloe vera gel had higher antioxidant activity during cold storage, which indicated by TEAC and FRAP. However, antioxidant activity of control fruit decreased during storage (Fig. 3A-B). Serrano *et al.* (2006) observed that Aloe vera gel treatment increased antioxidant activity in grape. It was supposed that antioxidant activity of Aloe vera gel is related to aloe-emodin, a hydroxyanthraquinone present in Aloe vera gel leaves and extracts (Serrano *et al.,* 2006). Therefore, higher antioxidant capacity observed in Aloe vera gel treated fig fruit could be related to the biochemical components of Aloe vera extract. Association was stated between the bioactive compounds and antioxidant activity, with the coated samples recording higher antioxidant activity (Anraku *et al.,* 2011).

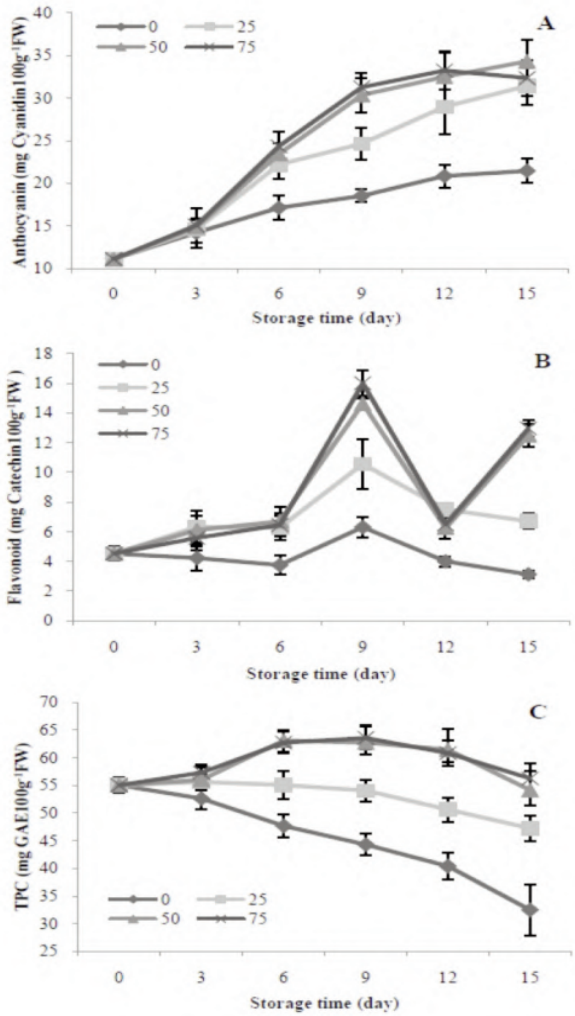

Fig. 2 - Anthocyanin (A), flavonoid (B) and total phenolic concentrations (TPC) (C) values in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%). Fruit were stored at 2°C. Vertical bars represent standard error of means of three experiments with four replicates per experiment.

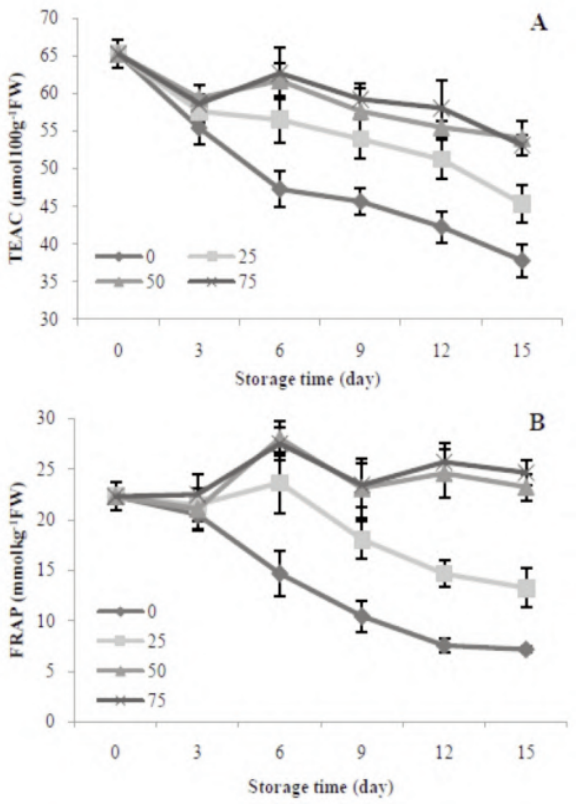

Fig. 3 - Antioxidant capacity with trolox equivalent antioxidant capacity (TEAC) (A) and ferric reducing antioxidant power (FRAP) (B) in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%) during cold storage at 2°C. Vertical bars represent standard error of means of three experiments with four replicates per experiment.

PAL and SOD

PAL activity in treated fruits increased during storage when compared with the control (Fig. 4). This indicates that Aloe vera gel treatment activated enzymes that are important in biosynthetic pathways of secondary metabolites of fruit. PAL is the first enzyme in phenylpropanoid pathway which catalyzes conversion of phenylalanine to trans-cinnamic acid and plays an important role in phenolic compounds biosynthesis (Hassanpour, 2015). PAL connects primary metabolism (shikimic acid pathway) to secondary metabolism (phenylpropanoid pathway) (Razavi and Hajilou, 2016). The results are comparable with Hassanpour (2015) who demonstrated that PAL activity in raspberry fruit increased when treated with Aloe vera gel. We suggest that Aloe vera gel treatment might be an efficient strategy for maintaining phenolic content in fig fruit via activation of PAL.

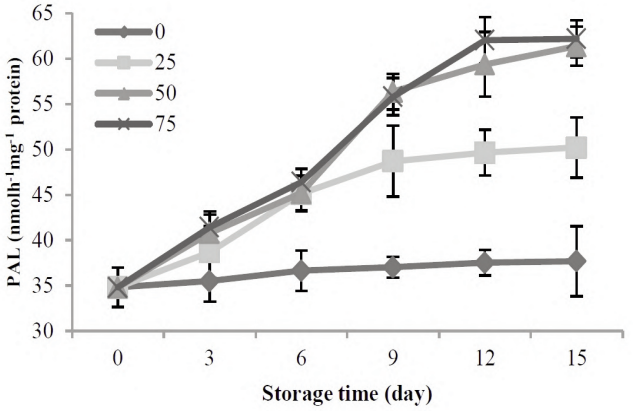

Fig. 4 - Phenylalanine ammonia-lyase (PAL) activity in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%) during cold storage at 2°C. Vertical bars represent standard error of means of three experiments with four replicates determinations per experiment.

SOD activity of non-treated figs was reduced during storage. However, SOD activity increased from day 3 to 6 in cold storage and decreased afterward when treated with Aloe vera gel (Fig. 5). Superoxide dismutases (SODs) which are metalloenzymes, are believed to play a crucial role in antioxidant defense because they catalyze the dismutation of O_2^- to H_2O_2. However, defensive action of SOD against O_2^-shows age-related changes. Higher SOD activity of Aloe vera coating treatments have been associated with cold storage stress tolerance fruit because it neutralizes the reactivity of the superoxide radical, which is over produced under stress (Bowler *et al.,* 1992). These results are comparable with Sun *et al*. (2010) who reported that SOD activity in litchi fruit treated with chitosan was higher than control fruit. These results have suggested that Aloe vera gel treatment might maintain TPC, FC and anthocyanin of fig fruit by increasing activity PAL and SOD enzymes.

Sensory evaluation

According to the judges, postharvest Aloe vera application did not have any negative effect on texture, taste and overall quality of fig (Fig. 6). The positive effect has an important role for consumers to buy the fruit. These results are in agreement with Song *et al.,* 2013 in which Aloe vera gel coated fresh cut apple had higher score than un-coated fruits.

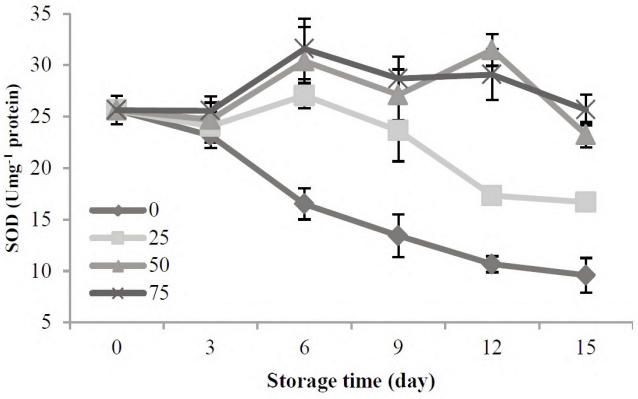

Fig. 5 - Superoxide dismutase (SOD) activity in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%) during cold storage at 2°C. Vertical bars represent standard error of means of three experiments with four replicates determinations per experiment.

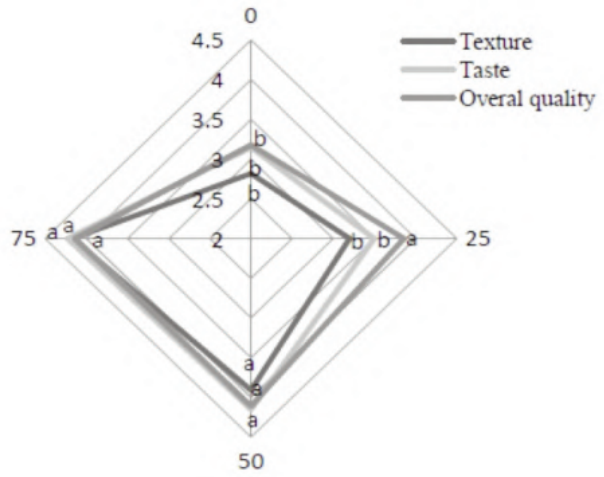

Fig. 6 - Sensory evaluation in figs (*Ficus carica* L.) 'Siyah' fruit treated with Aloe vera gel (0, 25, 50 and 75%) after transferring from 15 days in cold storage to the ambient temperature.

Thus, these results revealed that Aloe vera coting could decrease the loss of the sensory characteristics of fruit.

4. Conclusions

This research indicated that Aloe vera gel plays a positive role in maintaining TPC, anthocyanins, FC and antioxidant than control fruit. We conclude that Aloe vera gel treatment could be a useful alternative to the current chemical protocols for preserving nutraceutical traits of fig fruit by maintaining antioxidant capacity during storage.

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Effect of spread and shallow irrigation wetted area and application of organic mulch on citrus decline amelioration

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Key words: Citrus decline, fibrous root, irrigation, orange, root decay.

Abstract: Citrus decline threatens the orchards in the southern part of Iran. Roots begin to die-out before citrus decline deterioration. In this study, the effect of expanding the irrigation wetted area and decreasing the irrigation depth, and application of compost as organic mulch on root development and amelioration of citrus decline in Valencia orange trees (*Citrus sinensis* L. Osbeck) was investigated. Experimental factors contained the different percentage of irrigation wetted area and decreasing irrigation effective root depth by drip irrigation system at three levels - W0 (control): 30-40% with 60 cm effective root depth; W1: 50-60% with 45 cm effective root depth; W2: 70-80% with 30 cm effective root depth under tree canopy area and the factor of annual application of compost as organic mulch at two levels - M0 (control): means no application of compost and M1: application of 80 kg compost under tree canopy area with 10 cm thickness as ground cover, after rotating the top soil at 10 cm depth for all treatments. The results showed that, annual application of compost as organic mulch under tree canopy area and expanding the irrigation wetted area with decreasing irrigation effective root depth, significantly improved fibrous root length and density at a lower soil depth and decreased the indices of citrus decline such as root rot percentage, leaf and fruit drop and shoot die back. Also these treatments increased the water productivity and fruit quality of in declining Valencia orange trees.

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1. Introduction

Citrus decline, commonly known as 'dieback', 'chlorosis' or neglectosis, is not a specific disease but a syndrome expressing many disorders in the plant. Such syndrome leads to decline in productivity, reduced productive life and poor fruit quality. The symptoms of citrus decline contain root rot and blackening, shoot die back, growth stunt, fruit drop, reduction of canopy, smaller leaf number and size and leaf blotchy mottle (Meena *et al*., 2018). Stress-sensitive trees fail to maintain sufficient carbohydrate availability resulting in the dieback of the stressed tissues (Kreuzwieser and Rennenberg, 2014). Also, the percentage of total soluble solids and fruit juices in healthy trees is more than declined trees (Mauk and Shea, 2002). Declining trees have more water stress and more

affected trees tend to have higher percentage leaf and fruit drop rates than healthy appearing trees. Soil condition and water status (water stress and water logging) significantly reduce the citrus fibrous root density and increase the severity of citrus decline (Kozlowski, 1997; Graham *et al.*, 2013; Morgan, 2015; Graham, 2017).

Citrus decline is an issue that threatens the economy of various regions of the world, including southern Iran. There are several factors that affect the incidence of this complication. The most important influential factors on citrus decline are environmental stresses such as soil physicochemical conditions (compaction, high pH, bicarbonate, salinity) and soil nutritional status (Srivastava and Singh, 2009), soil moisture content (water stress and water logging with deficit or over irrigation), and biological stresses including rootstock, nematodes, Greening disease (Huanglongbing), Tristesa virus disease, phytophthora fungal infection and root fusarium (Johnson and Graham, 2015; Graham, 2017; Meena *et al*., 2018; Dewdney *et al*., 2019). According to the USDA, since 2004-2005 total Florida citrus production declined by Greening disease from 169.1 to 94.2 million boxes in 2015-2016, down 44.4% (USDA, 2017).

The ideal environment for citrus root development is a porous, medium-textured, well-drained soil, where water is easily available but not in excess (Dewdney *et al*., 2019). Mulching with organic matter helps retain moisture in the top soil by reducing surface evaporation, as well as moderating soil surface temperatures. It also enhances the decomposition process of soil organic matter and improve the soil aeration and availability of nutrients in the soil (Gong *et al*., 2006; FAO, 2011). Increasing root water uptake efficiency and life span is possible by irrigation management such as time and duration (decrease water stress and water logging), also improving the growth environment and drainage, soil compaction, soil thermal stress, bicarbonate and osmosis stress (Huber and Haneklaus, 2007; Dewdney *et al.*, 2019). Increased root density also increases water uptake (Morgan *et al*., 2006).

In arid and semi-arid regions, the application of drip irrigation system results in root accumulation under emitter (Fernandez *et al*., 1991; Tanasescu and Paltineanu, 2004; Ruiz-Sanchez *et al*., 2005), so the expansion of irrigation wetted area could enhance the fibrous root density under declining condition. Increasing irrigation frequency and decreasing irrigation depth stimulate root length density and increase water uptake and under these conditions, irrigation at the field capacity increases root density by 50% (Kadyampakeni *et al*., 2014 a).

In citrus decline new root growth did not stop, but the root survival time was reduced from 9 to 12 months to 4 months and the root decay and dieback were increased (Dewdney *et al*., 2019). Citrus decline cause water stress and under such condition root growth is preferable to shoot growth (Hsiao and Xu, 2000). Root development dependent on soil aeration and moisture as two fundamental parameters for root healthiness, growth and distribution. Root growth in sandy and loamy soils with higher organic matter content is stronger, and increment in soil clay content has a negative relationship with the density of citrus roots (Koudounas, 1994) and the slope of clay content in soil profile is directly related to the effect of citrus decline deterioration (Srivastava and Singh, 2009). In citrus, the highest fibrous length densities were observed in the humid part of the soil at a depth of 0 to 15 cm with higher root activity until 2 m horizontal distance from the trunk (Alves *et al*., 2012). The rate of soil water depletion is directly related to the abundance of fibrous roots and in general pattern of water uptake in citrus fruits indicates that water is drained by surface roots with higher amounts of available soil water (Noling, 2003).

Lack of soil aeration and excessive soil moisture causes more severe damage than lack of moisture in declining trees, due to stop breathing and the breakdown of root cells. There is also the growth of anaerobic microenvironments and the production of substances such as nitrite that is toxic for the roots. Centralize wetted area and excessive irrigation results in water logging, oxygen deficiency and root fungal infestationin the root zone (Johnson and Graham, 2015). Deficit irrigation and soil water stress stimulates longitudinal and singular growth without lateral roots and repeated moisture stress causes thicker fibrous roots in citrus that could decrease the water uptake efficiency by the citrus roots (Castle, 1978). Increment of soil aeration and soil moisture distribution and retention could provide the better condition for root development and amelioration of citrus decline. So in this experiment, the effects of spreading irrigation wetted area and decreasing irrigation depth, and annual application of compost as organic mulch on root development and diminishing of citrus decline indices in Valencia orange was investigated.

2. Materials and Methods

The experiment was conducted in a commercial orchard (28°38'13.12" N, 54°40'29.69" E and altitude 1138 m) of Darab region,with very hot and dry climate (Table 1), located in south west of Fars province in Iran, over three consecutive years 2016 to 2018. Ninty-six uniform 12-year-old Valencia orange trees (*Citrus sinensis* L. Osbeck), on lime (*Citrus aurantifolia*) rootstock with citrus decline symptoms (sparse foliage, chlorotic leaves, twig drying, premature leaf fall, reduced productivity and fruit size and die-back canopy complication) were selected based on canopy diameter and labeled based on experimental plan. The distance between the trees was 4×5 m and irrigated by drip irrigation system with a loop contained 6 emitters (4 liters per hour).

Before the application of experimental treatments, a composite soil sample were taken from 0-30 and 30-60 cm depths in Oct. 2014 (Table 2). Soil samples characteristics were determined at analytical laboratory of Soil and Water Research Department, Fars Agricultural and Natural Resources Research and Education Center, Zarghan, Iran. The soil of the experimental field site was calcareous with high pH, high total neutralizing value, and low amount of organic carbon, available P and Mn (Table 2). The fertilizer application was conducted based on soil test and contained fertigation of ammonium sulfate (21-0-0-24S, 450 g tree^{-1}), potassium sulfate (0-0-53+17S, 150 g tree^{-1}) and triple superphosphate (450 g tree^{-1}), manganese sulfate (32Mn, 18S, 250 g tree^{-1}) and iron chelate (200 g tree^{-1} sequestrene 138-Fe EDDHA 6%, 150 g tree^{-1}).

A Factorial (3×2) experiment was conducted in a randomized complete block design with four replications and each plot with four declining trees over three years. Experimental factors contained the different percentage of irrigation wetted area and decreasing irrigation effective root depth by drip irrigation system at three levels as follows:

- W0 (control)= 30-40% with 60 cm effective root depth;
- W1= 50-60% with 45 cm effective root depth;
- W2= 70-80% with 30 cm effective root depth under tree canopy area.

Also, the factor of annual application of compost as organic mulch at two levels:

- M0 (control)= means no application of compost;
- M1= application of 80 kg compost with 10 cm thickness as ground cover under tree canopy area.

Table 1 - Long-term mean of climatic elements of the study area (Darab) synoptic meteorological station statistical period (1997-2018)

	Temperature (°C)					Relative humidity (%)			Precipitation (mm)			Evapo. (mm) (montly sum)	Sunny day (montly sum)	No. day frost	Max wind (m s^{-1})	
	Min	Max	Ave.	Abs. Min	Abs. Max	Min	Max	Ave.	Amount	No. rainy	Max daily				Direc. degree	Velo. m/s
April	11.5	26.4	19.0	3.8	34.7	26	70	48	32.50	6	43.70	182.0	276.0	0	253	13
May	16.7	33.6	25.2	9.4	41.6	16	51	34	6.46	2	24.80	286.6	331.4	0	240	15
June	21.6	39.3	30.4	15.6	44.4	11	37	24	1.10	1	9.60	382.8	356.6	0	200	14
July	25.5	41.7	33.6	15.4	46.5	12	38	25	0.92	1	5.80	428.3	341.3	0	171	15
August	26.2	40.9	33.6	19.2	45.2	14	40	27	5.76	2	20.50	422.3	339.8	0	207	16
September	21.9	38.5	30.2	16.2	42.6	14	44	29	0.82	1	4.70	336.0	327.5	0	180	10
October	16.0	33.6	24.8	7.6	38.8	16	48	32	0.32	1	2.60	228.4	308.0	0	226	10
November	10.4	26.5	18.5	2.0	33.5	22	62	42	8.48	2	50.20	138.2	269.6	0	249	8
December	5.7	20.1	12.9	-2.2	30.0	32	76	54	40.25	5	51.80	79.1	234.9	0	207	7
January	3.8	17.0	10.4	-2.6	25.6	36	81	58	64.77	6	65.40	64.4	226.1	3	205	8
February	5.0	17.7	11.3	-2.6	25.8	35	81	58	49.88	6	68.20	79.3	238.4	1	240	10
March	8.0	21.8	14.9	0.2	31.6	30	74	52	37.26	7	63.00	116.7	241.1	0	238	12

Table 2 - Soil characteristics in the experimental orchard

Soil depth	Bulk density (g cm^{-3})	Field capacity (%)	Wilting point (%)	EC (dS.m^{-1})	pH	TNV (%)	OC (%)	P (mg kg^{-1})	K (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Soil texture
0-30	1.25	19.5	10.3	1.32	8.15	35.2	0.76	7.3	264.5	0.76	3.12	0.57	Sandy loam
30-60	1.28	19.3	10.4	1.36	8.16	37.7	0.62	7.2	252.4	0.62	3.19	0.56	Sandy loam

Compost applied 50 cm away from the trunk of the tree and over the soil surface, after rotating the soil at 10 cm depth for all treatments in March of each year. The physico-chemical properties of the applied compost contained organic carbon (22%), total nitrogen content (1.5%), C/N ratio (15), P_2O_5 (0.8%), K_2O (0.7%), electrical conductivity (6.4 dS. m^{-1}), pH (7.6), moisture content (12%), density (380 $kg.m^{-3}$), particle diameter (6 mm). The amount of irrigation wetted area was adjusted by increasing the number of emitters (6, 9 and 12 per loop with 4 liters per hour discharge) and the irrigation interval was determined by placing tensiometers (Irrometer Tensiometer Model SR Manual Gauge 12 in) deep down at three irrigations effective root depth (60, 45 and 30 cm, respectively) according to the irrigation experimental factor. Each year in February, the composite soil sample were taken from 0-10 cm and the percentage of soil organic matter, porosity and bulk density (g cm^{-3}) were determined. The amount of organic matter was measured by weight loss of oven-dried (105°C) soil sample after loss on ignition at 400°C. To determine the porosity of the samples, divide the pore space volume by the total volume and multiply the result by 100. Soil bulk density was determined by the weight of dry soil divided by the total soil volume. The amount of consumed irrigation water was measured by contours in each treatment. Over the three seasons, the indices of citrus decline were determined on tagged main branches on each tree quadrates. At the late spring, new flush lengths and the percentage of shoot dieback were measured. Leaf and fruit drop percentage were determined by counting their number at two separate time (after June drops and pre-harvest time). Leaf samples (contain 100 leaves) were collected during July-August from fully expanded new flush sub-terminal leaves from non-fruiting from tagged branches. Total chlorophyll content was determined by the method worked out by Lichtenthaler (1987). Leaf fresh weight (W_f) were recorded quickly using an analytical balance (Mettler Toledo AL104, Switzerland). Then, they were dried at 120°C in a circulation oven for 20 minutes, and the temperature dropped to 80°C until the constant weight (dry weight, W_d) was reached. Then the leaf relative water content (RWC) was calculated as:

$$\text{Leaf relative water content (\%)} = [(Wf - Wd)/Wf] \times 100$$

Individualleaf areas (LA) were determined by Licor leaf area meter, and sample were dried and specific leaf weights, SLW (mg cm^{-2}) were measured as:

$$\text{Specific leaf weight (mg cm}^{-2}) = Wd/\text{LA}$$

Root sampling was carried out under emitters from depth of 30, 45 and 60 cm soil depth, based on the effective root depth considered in the irrigation experimental factor, by auger (diameter 9 cm and height 25 cm), in Early-August (Alves *et al*., 2012). After washing the root samples, length, weight and number of fibrous root with less than 0.2 cm diameter was determined by digital ruler and weight scale, and length density (cm cm^{-3}), density (mg cm^{-3}) and the percentage of decayed fibrous root were determined in the soil sample volume harvested by auger (1589.6 cm^3) (Alves *et al.*, 2012).

At the harvesting time total fruit yield per trees was determined by the scale. A random sample of 50 fruits per plot was provided to determine fruit diameter (average of two perpendicular diameters), total soluble solids, pH and titratable acidity of the juice. Total soluble solids (Brix) were determined by using hand refractometer (WYT portable model) and juice total acid was measured by titration method with 0.1 N sodium hydroxide until pH meter reads 8.2 (Graham, 2017). Fruit juice percentage was calculated by fruit juice weight divided by fruit total weight. Water productivity (kg m^{-3}) was calculated based on the yield of trees to the amount of consumed irrigation water. The data set was auto scaled before analysis. All the parameters for three years were subjected to combine analysis of variance (ANOVA) by MSTAT-C software. Means were compared by Duncan's multiple range test and Pearson correlation were determined by SPSS software.

3. Results

The results of the combine analysis of variance (ANOVA) over three consecutive seasonal growths from 2016 to 2018, indicated that there was a significant effect of experimental factors on improvement of citrus decline deterioration indices in Valencia orange (Tables 4-8). Annual application of compost as organic mulch and adding them to 10 cm top soil in the following years had significant effect on the increment of soil organic matter and soil porosity up to 170.45, 13.18% respectively, and decreasing soil bulk density by 11.98% at 10 cm soil depth in comparison with control (without application of compost as mulch) (Table 3).

Annual application of compost as organic mulch significantly increased the fibrous roots length density and fibrous roots density of Valencia orange trees by 34.63 and 38.27% respectively. Also the interac-

Table 3 - Effects of experimental treatments on 10 cm top soil organic matter, porosity and bulk density over three consecutive seasonal growths from 2016 to 2018

Treatments	Soil organic matter (%)	Soil porosity (%)	Bulk density (g cm-^{3})
W0M0	1.42 ± 0.12 b	42.05 ± 1.21 b	1.49 ± 0.04 a
W1M0	1.24 ± 0.14 b	41.86 ± 1.32 b	1.43 ± 0.04 a
W2M0	1.30 ± 0.10 b	42.42 ± 1.15 b	1.42 ± 0.05 a
W0M1	3.38 ± 0.07 a	47.08 ± 1.01 a	1.31 ± 0.06 b
W1M1	3.84 ± 0.08 a	48.51 ± 1.02 a	1.27 ± 0.07 b
W2M1	3.50 ± 0.07 a	47.39 ± 0.93 a	1.24 ± 0.06 b

Mean separation (± SD) within columns followed by different letters are significantly different at *P*≤0.05 using Duncan's new multiple range tests.

tion between expansion the percentage of irrigation wetted area along with and decreasing irrigation effective root depth with annual application of compost as organic mulch (W1M1 and W2M1), significantly increased the fibrous root density up to 87.09 and 112.9% and decreased the root decay by 43.23 and 46.48% at a lower soil depth respectively in comparison with control (W0M0) (Table 4).

Table 4 - Effects of experimental treatments on root characteristics of declining Valencia orange over three consecutive seasonal growths from 2016 to 2018

Treatments	Fibrous roots length density (cm cm -^{3})	Fibrous root density (mg cm-^{3})	Root decay (%)
W0M0	0.043 ± 0.003 d	0.31 ± 0.08 d	66.24 ± 6.21 a
W1M0	0.054 ± 0.002 c	0.48 ± 0.06 c	53.72 ± 8.60 b
W2M0	0.056 ± 0.002 c	0.49 ± 0.06 c	54.25 ± 7.32 b
W0M1	0.066 ± 0.003 b	0.53 ± 0.04 bc	48.07 ± 7.56 b
W1M1	0.065 ± 0.004 b	0.58 ± 0.04 b	37.60 ± 8.14 c
W2M1	0.075 ± 0.003 a	0.66 ± 0.03 a	35.45 ± 8.81 c

Mean separation (± SD) within columns followed by different letters are significantly different at *P*≤0.05 using Duncan's new multiple range tests.

In comparison with control (without compost as mulch), application of compost as organic mulchsignificantly increased the total leaf chlorophyll content and new flushes length up to 36.3 and 29.67% respectively and decreased leaf and fruit drop (Table 5, 6) and shoot dieback by 40.21, 38.18 and 40.59%, respectively (Table 5), in declining Valencia orange trees, respectively. Increment the irrigation wetted area percentageand decreasing the effective root depth for irrigation, significantly increased leaf chlorophyll contentand flush lengthand reduced leaf and fruit drop and shoot dieback. The interaction between the expansionof irrigation wetted area with annual application of compost as organic mulch (W1M1 and W2M1) had the highest impact on the increment of total leaf chlorophyll contentup to 58.62 and 62% and flush length by 75.78 and 80.9% and decreasing leaf drop 56.17 and 71.1%, fruit drop by 44.62 and 63.3% and shoot dieback by 56.3, 76.44%, respectively, in comparison with control (W0M0).

Leaf relative water content, specific leaf weight, fruit diameter and tree yield increased with annual application of compost as organic mulchanddeveloping the irrigation wetted area (Table 6). The interaction between irrigation wettedarea and annual application of compost as organic mulch (W1M1 and W2M1) had the highest impact on the increment of leaf relative water content up to 11.91 and 14.25% and specific leaf weight up to 18.75 and 22.66%, respectively, in comparison with control (W0M0) (Table 6). Also Fruit diameter significantly increased by increment of wetted area and application of mulch by 15.9 and 14.11%, respectively (Table 6).

The highest yield of Valencia orange trees was belonged to the interaction between irrigation wetted area of 70-80% with 30 cm effective root depth for irrigation and application of compost mulch

Table 5 - Effects of experimental treatments on vegetative growth indices of declining Valencia orange over three consecutive seasonal growths from 2016 to 2018

Treatments	Total leaf chlorophyll content (mg g-^{1} FW)	Flush length (cm)	Leaf drop (%)	Shoot dieback (%)
W0M0	0.29 ± 0.03 d	24.82 ± 2.31 d	35.30 ± 1.12 a	35.70 ± 2.11 a
W1M0	0.34 ± 0.02 c	35.53 ± 3.78 c	27.63 ± 1.53 b	29.62 ± 3.10 b
W2M0	0.36 ± 0.01 c	38.35 ± 3.81 b	21.30 ± 1.71 c	17.60 ± 2.27 d
W0M1	0.42 ± 0.03 b	39.47 ± 3.15 b	24.70 ± 1.92 bc	25.25 ± 2.15 c
W1M1	0.46 ± 0.02 a	43.63 ± 2.80 a	15.45 ± 1.65 d	15.60 ± 2.84 d
W2M1	0.47 ± 0.02 a	44.90 ± 2.66 a	10.20± 1.86 e	8.41 ± 1.90 e

Mean separation (± SD) within columns followed by different letters are significantly different at *P*≤0.05 using Duncan's new multiple range tests.

(Table 7). Annual application of compost as organic mulch had significant effect on the reduction of consumed irrigation water by 17.39% in comparison with control (without mulch). The interaction between irrigation wetted area and annual application of compost as organic mulch (W1M1 and W2M1) had the highest impact on the increment of water productivity up to 53.06 and 49.79% respectively, in comparison with control (W0M0), and there was no significant difference between them (Table 7). Expansion of irrigation wetted area and decrease effective root depth for irrigation under the condition of compost mulch applicationhad the greatest effect on water productivity in Valencia orange.

Annual application of compost as organic mulch significantlyincreased the fruit juice content by 11.98%, total soluble solids (Brix) by 10.96%, Brix/Titratable acidity ratio by 24.52% in declining Valencia orange trees. Fruit acidity was significantly reduced by annual application of mulch. Increment of irrigation wetted area percentage significantly, increased fruit juice and brix/titratable acidity ratio. The interaction between increased irrigation wetted area with mulch application (W1M1 and W2M1), resulted in the highest increase in fruit juice percentage by 35.43 and 34%, total soluble solidsby 23 and 26.36% and brix/titratable acidity ratio by 49.6 and 57.6%, respectively in comparison with control (W0M0) (Table 8).

Table 6 - Effects of experimental treatments on the leaf relative water content, specific leaf weight, fruit drop and fruit diameter of declining Valencia orange over three consecutive seasonal growths from 2016 to 2018

Treatments	Leaf relative water content (%)	specific leaf weight (mg cm-^{2})	Fruit drop (%)	Fruit diameter cm)
W0M0	83.50 ± 5.30 d	3.84 ± 0.12 d	31.15 ± 1.10 a	7.23 ± 0.10d
W1M0	89.64 ± 5.24 c	4.12 ± 0.08 c	25.12 ± 1.32 b	7.58 ± 0.08 c
W2M0	91.25 ± 4.22 bc	4.36 ± 0.07 bc	21.84 ± 1.25 bc	8.20 ± 0.07 b
W0M1	88.36 ± 5.75 c	4.04 ± 0.09 cd	19.61 ± 1.74 c	7.71 ± 0.08 c
W1M1	93.45 ± 4.12 ab	4.56 ± 0.06 ab	17.25 ± 2.11 c	8.38 ± 0.06 a
W2M1	95.48 ± 4.53 a	4.71 ± 0.06 a	11.43 ± 2.73 d	8.25 ± 0.07 ab

Mean separation (± SD) within columns followed by different letters are significantly different at $P \leq 0.05$ using Duncan's new multiple range tests.

Table 7 - Effects of experimental treatments on the leaf characteristics, fruit quantity and water productivity of declining Valencia orange over three consecutive seasonal growths from 2016 to 2018

Treatments	Total tree yield (kg)	Consumed irrigation water (m^{3} ha-^{1} year-^{1})	Water productivity (kg m-^{3})
W0M0	62.33 ± 2.14 e	11625.28 ± 136.58 c	2.45 ± 0.11 c
W1M0	71.05 ± 2.71 d	13857.14 ± 122.61 b	2.39 ± 0.16 c
W2M0	83.10± 1.50 c	14631.67 ± 121.25 a	2.66 ± 0.14 bc
W0M1	68.47 ± 2.43 d	10363.64 ±136.84 d	3.01 ± 0.12 b
W1M1	89.40± 1.37 b	10904.76 ± 132.70 d	3.75 ± 0.11 a
W2M1	94.40± 1.15 a	11869.57 ± 126.39 c	3.67 ± 0.12 a

Mean separation (± SD) within columns followed by different letters are significantly different at $P \leq 0.05$ using Duncan's new multiple range tests.

Table 8 - Effects of experimental treatments on the fruit quality indices of declining Valencia orange over three consecutive seasonal growths from 2016 to 2018

Treatments	Fruit juice (%)	Titratable acidity (g 100-^{1} ml)	Total soluble solids (Brix)	Brix/Titratable acidity ratio
W0M0	42.64 ± 2.02 d	1.16 ± 0.01 a	9.56 ± 0.61 d	8.94 ± 0.22 f
W1M0	48.80 ± 1.81 c	0.94 ± 0.03 b	11.13 ± 0.35 c	10.26 ± 0.19 e
W2M0	53.71 ± 1.60 b	0.95 ± 0.02 b	11.87 ± 0.24 b	11.87 ± 0.17 c
W0M1	47.65 ± 2.17 c	0.82 ± 0.02 c	12.29 ± 0.73 a	11.23 ± 0.18 d
W1M1	57.75 ± 1.61 a	0.74 ± 0.03 d	11.76 ± 0.40 b	13.37 ± 0.15 b
W2M1	57.14 ± 1.55 a	0.84 ± 0.03 c	12.08 ± 0.37 b	14.09 ± 0.14 a

Mean separation (± SD) within columns followed by different letters are significantly different at $P \leq 0.05$ using Duncan's new multiple range tests.

Results showed that fibrous root density had a significant negative correlation with leaf drops (r= -0.791) and shoot dieback (r= -0.612) (Table 9). Also fibrous root density had positive and significant correlation with flush length (r= 0.624), leaf water content (r= 0.732) and specific leaf weight (r= 0.631). The root decay percentage had significant negative correlation with leaf chlorophyll content (r= -0.643), flush length (r= -0.632), leaf water content (r= -0.603), leaf specific weight (r= -0.638), yield (r= -0.691), water productivity (r= -0.602), and Brix/TA (r= -0.684). Also there was a positive and significant correlation between the percentage of root decay with leaf drops (r= 0.784), shoot dieback (r= 0.692), and fruit drops (r= 0.704) (Table 9). Specific leaf weight had positive and significant correlation with leaf chlorophyll content (r= 0.482) (Table 9). There was a negative correlation between fruit drops and relative water content of leaves (r= -0.774). Tree water productivity had a positive and significant correlation with the leaf chlorophyll content (r= 0.612), leaf relative water content (r= 0.766), and specific leaf weight (r= 0.677) (Table 9).

4. Discussion and Conclusions

Citrus decline was directly related to root decay and decreasing of fibrous root density and root expansion in Valencia orange trees. Tree performance is a function of how the root system is distributed over a large volume of soil to absorb water and nutrients (Lehmann, 2003). In arid and semi-arid regions, the highest density of fibrous roots in the drip irrigation system is located under the emitters and soil wetted area (Ciancio and Mukerji, 2008; Alves *et al*., 2012). The rate of water adsorption by the tree is reduced when the soil oxygen level is low (Levy, 1998; Boman *et al*., 1999) and the most influential soil condition on citrus decline are soil moisture condition (Meena *et al*., 2018), and soil compaction (Srivastava and Singh, 2009). Root development is related to the hydrophilicity of roots, soil aerationand distribution of soil moisture (Ruiz-Sanchez *et al*., 2005).

Our results showed that Application of compost as organic mulch and rotating them with 10 cm topsoil improved soil physical properties such as soil organic matter, porosity and bulk density which could provide the best conditions for root development. This treatment could increase the soil aeration and soil moisture retention at topsoil, as two key factors for root health and growth (Boman and Parsons, 2002; Nelson *et al.*, 2008; Johnson and Graham, 2015). Meanwhile, it could reduce surface evaporation, moderate soil surface temperatures and provide the best condition for root development for declining Valencia orange trees. Application of organic mulches on Eureka lemon (*Citrus limon* Burm) significantly increased the soil moisture status in various soil

Table 9 - Pearson correlation coefficients between the means of citrus decline indices of Valencia orange over three consecutive seasonal growths from 2016 to 2018

	Chlorophyll content	Flush length	Leaf drop	Shoot dieback	LRWC [1]	SLW [2]	Fruit drop	Fruit diameter	Yield	Water productivity	Brix/TA.	Fibrous roots density	Fibrous roots length density	Root decay
Chlorophyll content	1.00													
Flush length	0.64 **	1.00												
Leaf drop	-0.51 *	-0.64 **	1.00											
Shoot dieback	-0.55 *	-0.74 **	0.78 **	1.00										
LRWC	0.54 *	0.64 **	-0.77 **	-0.75 **	1.00									
Fruit drop	-0.66 **	-0.25 NS	0.67 **	0.64 **	-0.71 **	-0.69 **	1.00							
Fruit diameter	0.43 *	0.49 *	-0.52 *	-0.51 *	0.52 *	0.53 *	-0.53 *	1.00						
Yield	0.59 **	0.65 **	-0.76 **	-0.67 **	0.66 **	0.60 **	0.26 NS	0.75 **	1.00					
Water productivity	0.61 **	0.50 *	-0.75 **	-0.65 **	0.77 **	0.68 **	-0.75 **	0.78 **	0.78 **	1.00				
Brix/T.A.	0.63 **	0.52 *	-0.70 **	-0.68 **	0.15 NS	0.57 **	-0.66 **	0.13 NS	0.57 **	0.46 *	1.00			
Fibrous roots density	0.71 **	0.62 **	-0.79 **	-0.61 **	0.73 **	0.63 **	-0.71 **	0.64 **	0.70 **	0.71 **	0.62 **	1.00		
Fibrous roots length density	0.58 *	0.48 *	-0.59 **	-0.54 *	0.68 **	0.59 **	-0.54 *	0.61 **	0.62 **	0.59 **	0.52 *	0.78 **	1.00	
Root decay	-0.64 **	0.63 **	0.78 **	0.69 **	-0.60 **	-0.64 **	0.70 **	-0.66 **	-0.69 **	-0.60 **	-0.68 **	-0.64 **	-0.71 **	1.00

[1] Leaf relative water content.
[2] Specific leaf weight.
*, ** significant at 5 and 1% statistical levels respectively; NS = not significant.

depths and farmyard manure were found to be more effective in producing maximum growth extension (Kumar *et al.*, 2015). The irrigation wetted area, also affect citrus root system development and distribution (Alves *et al.*, 2012). In our experiment, the expansion of irrigation wetted area and reduction the effective root depth for irrigation with annual application of compost as organic mulchand its rotation in the 10 cm soil depth, increased the fibrous root length and root densityat lower soil depths and decreased root decay in Valencia orange trees. Also, leaf relative water content, specific leaf weight and fruit diameter in Valencia orange significantly increased with developing the irrigation wetted area. Increment of fibrous root length and root density at lower soil depths with decreasing the irrigation depth and consequent improvement of tree water status were in agreement with the results that showed more root distribution in the drip irrigation method was at the depth of 15 cm, with high water uptake efficiency (Kadyampakeni *et al.*, 2014 b). Annual application of compost as organic mulch significantly decreased the consumed irrigation water in Valencia orange trees. Increasing soil organic carbon improve the healthy root system of citrus (Sharma *et al*., 1986). There was a positive and significant correlation between leaf relative water content and leaf chlorophyll content (r= 0.540), and flush lengths (r= 0.643) and a significant negative correlation between leaf water content and leaf drops (r= -0.771), and shoot dieback (r= -0.748) (Table 8). It has been shown that clementine 'Nules' vegetative growth and fruit size was higher with increasing numbers of emitters on the double drip-lines treatments (Abouatallah *et al*., 2012).

We conclude that annual application of compost as organic mulch under tree canopy area and its rotation at 10 cm soil depth at following years and expanding the irrigation wetted area and decreasing the irrigation effective root depth, significantly improved the indices of citrus decline and increased the water productivity and fruit quality in Valencia orange trees.

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Feasibility of Vis/NIR spectroscopy to detect and estimate fungicide residues on intact lettuces

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Key words: dithiocarbamate, *Lactuca sativa* L., multivariate data analysis, spectral reflectance.

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Abstract: Pesticides are applied repeatedly to grain, fruit, and vegetable crops for protection against pathogens, pests, and weeds, in short periods of time before harvest. The effective and fast monitoring of chemical residues in agricultural products is important for the assurance of healthy food. This study was accomplished to evaluate the feasibility to detect and estimate the concentration of dithiocarbamate fungicide (mancozeb) residues on intact lettuce leaves based on Vis/NIR spectral reflectance measurements and multivariate data analysis. In the pre-harvest interval, a high initial rate of decline of dithiocarbamate residues was observed between one and seven days after pesticide spraying (decrease of 90.3%), while a slower decline was verified from seventh to fourteenth day (decrease of 8.7%). The usefulness of this spectrometric method has been evidenced by determination of dithiocarbamate residues at concentrations between 0.23 and 10.3 mg CS_2 kg^{-1}, with detection and quantitation limits of 0.49 and 1.41 mg CS_2 kg^{-1}, respectively. Vis/NIR spectral reflectance combined to the partial least square analysis have potential to be applied for estimating dithiocarbamate concentrations on intact lettuce leaves, presenting advantages such as real-time measurements and the possibility to be built into the industrial processing lines.

1. Introduction

Consumers demand grain, fruits, and vegetables with high sensorial and nutritional qualities, but without pesticides. Although the correct use of fungicides does not cause problems of public concern in health and environmental areas, undesirable residues can remain on agricultural products after harvest if inappropriate or abusive treatments are applied without respecting safety recommendations indicated by the specialized agencies and manufacturers (López-Fernández *et al.*, 2013).

In modern agriculture, pesticides are applied repeatedly to grain, fruit, and vegetable crops for protection against pathogens, pests, and weeds, in short periods of time before harvest (Jankowska *et al.*, 2019). The main exposure to pesticides for humans is via food, especially by consumption of agricultural products.

Introduced between 1940 and 1980, dithiocarbamate fungicides still represent an important class of pesticides widely used in agriculture. They are characterized by a broad spectrum of activity against various plant pathogens, low acute mammal toxicity, and low production costs (Crnogorac and Schwack, 2009). However, laboratory studies showed that dithiocarbamates can result in neuropathology, thyroid toxicity, and developmental toxicity to the central nervous system (IPCS, 1993; Caldas *et al.*, 2006).

Mancozeb is an ethylene-bis-dithiocarbamate used as fungicide to protect fruit and vegetable crops from a range of fungal diseases (Pereira *et al.*, 2014) and was considered a multipotent carcinogenic agent in a long-term study (Belpoggi, 2002). This fungicide is registered to 38 food crops, occupy the third place in the ranking of the most commercialized pesticides in Brazil in terms of tones of active ingredient (ANVISA, 2018; IBAMA, 2018).

Caldas *et al.* (2006) reported data obtained from the Program on Pesticide Residue Analysis in Food (PARA), coordinated by the Brazilian National Sanitary Surveillance Agency (ANVISA), about dithiocarbamate residues on 34% of lettuce samples in a total of 297 analyzed, based on vegetable samples collected from 2001 to 2004. In another Brazilian study, accomplished from 2005 to 2015, Jardim *et al.* (2018) showed that 14.6% of 1483 lettuces presented dithiocarbamate residues. López-Fernández *et al.* (2013) verified the presence of mancozeb and other dithiocarbamate residues on 72% of lettuce samples collected at Spain, with some samples containing residues three times higher than the maximum limit (5 mg kg^{-1}).

Various methods have been improved for the determination of dithiocarbamate residues, including gas and/or liquid chromatography, often in conjunction with mass spectrometry (Crnogorac and Schwack, 2009). These current methods requiring sample preparation (destructive), time consuming, and laboratory labor demanding, well-trained personnel and relatively expensive process chemistry. They also produce chemical and sample waste, which adversely affects product traceability by preventing real-time decision-making (Salguero-Chaparro *et al.*, 2013; Steidle Neto *et al.*, 2017). Although more sensitive, these methods are suitable for spot checks.

The reasons for Vis/NIR spectroscopy great success as one of the most important and versatile techniques in analytical chemistry include its speediness and easiness to handle and provide molecular specific information for different types of samples in any physical state, with little or no previous chemical treatment (Gonzálvez *et al.*, 2011). However, one possible drawback is the range of concentration of the target analyte. Pesticide residues tend to have very small concentrations in foods. Despite this, previous studies proved the feasibility of spectroscopy to detect and quantify low concentrations of analytes in fruits and vegetables (Saranwong and Kawano, 2005; Gonzálvez *et al.*, 2011; Acharya *et al.*, 2012). Nevertheless, very few published studies have addressed the use of Vis/NIR spectroscopy for predicting pesticides residues in harvested intact samples. The scientific researches of Sánchez *et al.* (2010), for peppers, and Jamshidi *et al.* (2016), for cucumbers, reported estimates of pesticides without pre-treatment or preparation of samples. This advantage allows that spectroscopy technique can be built into the processing line, enabling large-scale individual analysis and real-time decision-making. Another recent technique for trace level detection of pesticides is the Surface Enhanced Raman Spectroscopy (SERS), that use noble metal nanostructures (e.g. gold) to increase the weak signals from analytes. But, SERS requires spectrometer, laser source, probe, sample holder, and substrates that are more expensive when compared with spectral reflectance equipment.

The present scientific research was carried out to evaluate the feasibility to detect and estimate the concentration of dithiocarbamate fungicide residues on intact lettuce leaves based on Vis/NIR spectral reflectance measurements and multivariate data analysis.

2. Materials and Methods

Lettuce cultivation

Lettuce (*Lactuca sativa* L. cv. Regina) with plain green leaves, was cultivated under organic conditions in a certified farm located at the Capim Branco city, Minas Gerais State, Brazil (19° 34' S latitude, 44° 10' W longitude, and 816 m a.s.l.). According to Köppen classification, the region climate is Cwa (warm temperate - mesothermal), with dry winter and rainy summer.

The lettuce seeds were planted in plastic trays containing organic substrate. Seedling production occurred under a low-density polyethylene cover (Suncover Av Blue 120 µm, Ginegar Polysack, São Paulo, Brazil), which allowed a better irrigation control and was internally coated with a photoselective

shading mesh (ChromatiNet Raschel Red 35%, Ginegar Polysack, São Paulo, Brazil), capable of reducing the percentage of beam solar radiation on the plants, also increasing the fresh mass production.

The vigorous and healthy seedlings were individually transplanted to plastic pots containing a thin gravel layer overlapped by soil mixed with organic compound (cattle manure and vegetable biomass). The crop irrigation was performed by a drip system, controlled by a digital timer. The experiment consisted of 500 lettuce plants. However, 355 plants were effectively used as experimental units and 145 plants were cultivated for boundary effects.

Some days before plants reach the physiological maturation, the lettuces were transported to a greenhouse with the objective of performing the fungicide spraying. The greenhouse is located at the Campus Sete Lagoas of the Federal University of São João del-Rei, which is distant from the organic farm about 22 km. This greenhouse also was covered and coated with the same polyethylene film and photo selective shading mesh described above.

Fungicide spraying

A non-systemic fungicide (mancozeb) was used, which is classified in the alkylenebis group (dithiocarbamate). The active ingredient of this pesticide is a Carbon Disulphide (CS_2) precursor and is registered in the Brazilian Ministry of Agriculture, Livestock, and Food Supplies (MAPA) for application on some crops. However, the dithiocarbamate (mancozeb) is not authorized by the Brazilian National Sanitary Surveillance Agency (ANVISA) for lettuce crop. This fungicide was chosen based on reports of the Brazilian Monitoring Program for Pesticides Residues in Food, developed by ANVISA, that mentioned the indiscriminate use of this pesticide on lettuces and other crops by some Brazilian farmers. Contrarily, this fungicide is authorized for lettuce crop by other agencies and committees, such as the European Food Safety Authority and the FAO Codex Alimentarius.

The solubilization of mancozeb in water was done to provide a sufficient volume for uniform and homogeneous application on lettuces. The dosage prescribed in the Brazilian package leaflet for other green leafy vegetables, such as cabbage, was adopted (2-3 kg of fungicide ha^{-1}). This dose is recommended to control the mildew (*Peronospora parasítica*) and the pod spot (*Alternaria brassicae*). Excepting for five plants (control units), which were randomly selected in the greenhouse, the application of mancozeb to lettuces was performed using an electric sprayer (droplets with average diameter of 29 µm). The application time and distance from the sprayer nozzle to the plants were standardized.

Similar to the dosage, the pre-harvest interval for consumption of green leafy vegetables informed in the Brazilian package leaflet (14 days) was considered in this study. Samplings for the spectral reflectance measurements and laboratory analyzes were carried out on alternate days during the pre-harvest interval, totaling 7 days and starting one day after the fungicide spraying.

Daily 10 samples were collected, each one weighting more than 500 g and corresponding to five lettuce plants randomly selected. The fresh mass was determined using a precision balance and discarding the roots. Therefore, 50 plants were used per day, totaling 350 plants during the sampling period.

The statistical design was entirely randomized with 7 treatments (alternate days after spraying) and 10 repetitions (samples).

Reflectance measurements

Spectral reflectance was measured by a miniature and hand-held spectrometer (JAZ-EL350, Ocean Optics, Dunedin, USA), coupled to a tungsten-halogen light source, and preconfigured to acquire and store reflectance data from 350 to 1000 nm, with spectral resolution of 1.3 nm. A specific clip probe (SpectroClip-R, Ocean Optics, Dunedin, USA) was used to collect reflected light from the lettuce leaves (Fig. 1). This probe contains an integrating sphere that captures diffuse reflected light more efficiently

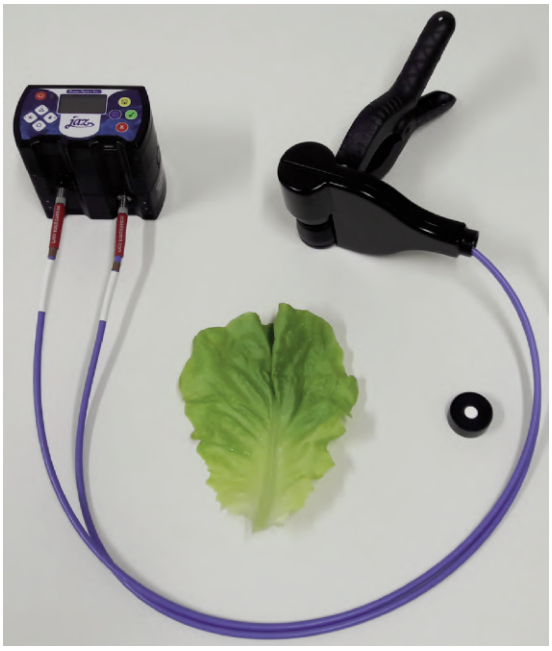

Fig. 1 - Hand-held spectrometer, clip probe, and diffuse reflectance standard used to collect reflected light from the lettuce leaves.

than lens-based collection optics. The active illuminated leaf area in the clip probe is 5 mm diameter. Two premium fibers Vis/NIR (600 µm) interconnected the spectrometer and the light source to the clip probe. A diffuse reflectance standard with Spectralon™ was used as a reference to measure spectral reflectance.

After the warm up time of the light source, the reference standard measurements were made before the spectral reflectance measurements on lettuce leaves. Data acquisitions were performed in a temperature-controlled environment with the purpose of avoiding the overheating of the light source and the spectrometer detector due to the extended using time. The reflectance values were calibrated by means of the software (OceanView, Ocean Optics, Dunedin, USA) and expressed as a relative percentage of the reference standard (Xing *et al.*, 2006):

$$R_{\lambda}^{cal} = [(R_{\lambda}^{leaf} - R_{\lambda}^{dark})/(R_{\lambda}^{ref} - R_{\lambda}^{dark})] \times 100 \quad (1)$$

where R_{λ}^{cal} is the calibrated spectral reflectance from the leaves (%), R_{λ}^{leaf} is the spectral reflectance from the leaves (dimensionless), R_{λ}^{dark} is the spectral reflectance considering light absence (dimensionless), and R_{λ}^{ref} is the spectral reflectance from the diffuse reflectance standard (dimensionless).

Three leaves of each plant were randomly selected from the external, middle, and central parts of the lettuce head. Three separate measurements on standardized and equidistant locations of the adaxial surface were performed in each leaf, avoiding its central vein and boundaries. Thus, 3195 spectral signatures were obtained during the sampling period, considering the 350 sprayed plants and the five control lettuces (without dithiocarbamate).

The electronic files containing the spectral signatures were stored in a memory card and later transferred to a notebook for analyzes performed with electronic spreadsheets. During the analyzes, spectral signature averages were obtained for each lettuce sample. After this, data was stored in an external hard disk.

Dithiocarbamate analytical determination

Samples were quartered, milled in an electric grinder, placed in hermetic packages, and frozen at -30°C in an ultrafreezer for minimizing the degradation and metabolization of the dithiocarbamate.

The analytical determination of the concentration of dithiocarbamate was performed based on the method proposed by Cullen (1964) and improved by Keppel (1971). Mancozeb residues were measured by the spectrophotometric determination of the cupric complex formed with the CS_2 evolved from the acid decomposition of dithiocarbamate in the presence of stannous chloride as a reducing agent (Caldas *et al.*, 2004). The solution of the complex formed from the reaction between CS_2 and copper (II) acetate monohydrate was measured at 435 nm in UV/Vis spectrophotometer (Cary 50, Varian, Agilent Technologies Inc., USA). At the end of the laboratory analyzes, 70 reference measurements were obtained, corresponding to 10 values for each treatment.

Data analysis

The Partial Least Squares (PLS) multivariate analysis was applied with the purpose of developing a mathematical model capable of predicting the dithiocarbamate concentrations based on lettuce pretreated spectral signatures. Thus, a response matrix, composed by the dithiocarbamate concentrations obtained by laboratory analytical measurements, was correlated with a spectral matrix, containing the average reflectance measurements. An orthogonal basis of latent variables was constructed one by one in such a way that they were oriented along the directions of maximal covariance between the two original spaces (response and spectral matrices), trying to achieve an optimal prediction for new data (Wold *et al.*, 2001; Anderson, 2009; Cozzolino *et al.*, 2011).

The latent variables were calculated by iterative methods as linear combinations of the original independent variables (spectral reflectances) and the dependent ones (dithiocarbamate concentrations). New variables were found, representing estimates of the latent variables or their rotations. These new variables were called X-scores and were predictors of the response ones. A weight matrix was also calculated so that each of their elements maximized the covariance between response variables and the corresponding latent variable scores. The unexplained part of the predictor variables was represented by the deviations between the measured and predicted responses, which were also calculated and called Y-residuals (Wold, 2001; Lopes and Steidle Neto, 2018).

The detrending pre-treatment was applied to the spectral signatures prior to the model calibration and external validation. The detrending algorithm corrected scatter and simple deformations of the spectra baseline as vertical shift and slope (Barnes *et al.*, 1989; Steidle Neto *et al.*, 2016). According to Moura *et al.* (2016), this was the most effective pre-treatment for removing irrelevant information which could not be handled by the regression technique and principal component analysis.

As recommended by Huang *et al.* (2008), two thirds of the data for each response variable were used as the calibration with cross-validation set, whereas one third of the data as the external validation set. The calibration set was used for developing the model and the external validation set was employed for assessing the calibrated model prediction performance. This procedure was applied for predicting independent data, not related with those included in the calibration with cross-validation set (Agelet and Hurburgh, 2014). During calibration the leave-group-out cross-validation technique was applied to data. This sampling plan also agrees with the suggested by Kramer (1998), who affirmed that the number of data in the calibration set should be more than 10 times the number of variable components in the experiment. In this study the dithiocarbamate residues represented the independent source of significant variation in the data.

The software SPECTOX was specifically developed for this study with the purpose of assisting in the spectral reflectance data processing and the multivariate analysis (calibration and validation), also allowing the performance evaluation of the prediction model for dithiocarbamate concentrations. The SPECTOX was written in Java language, by using the free NetBeans IDE (Apache Software Foundation, Maryland, USA). Additional algorithms of the pretreatments and multivariate methods were included in the SCILAB software (Scilab Enterprises, Versailles, France).

The optimal number of latent variables was determined as recommended by Jha (2010), considering the minimum value for the root mean square error to avoid over fitting (Eq. 2).

$$RMSE = \sqrt{\frac{\sum (Y_o - Y_p)^2}{n}} \quad (2)$$

Where RMSE is the root mean square error (mg kg^{-1}), Y_o are the values measured by the UV/Vis spectrophotometer (mg kg^{-1}), Y_p are the values predicted by the model (mg kg^{-1}), and n is the number of samples (dimensionless).

The calibration and cross-validation processes were evaluated by the root mean square errors for calibration (RMSEC) and cross-validation (RMSECV) sets, respectively. Additionally, the predictive capacity of the adjusted model regarding external validation was evaluated by the statistical parameters mean absolute error (MAE), mean bias error (BIAS), coefficient of determination (R^2), and index of agreement (d).

Willmott and Matsuura (2005) pointed out that MAE (Eq. 3) is unambiguous and the most natural measure of the mean error magnitude. These authors considered that MAE should be used as the basis for all dimensioned evaluations and inter-comparisons of model performance. The BIAS (Eq. 4) represents the average difference between measured and predicted data. Thus, values close to zero indicate low systematic error between the measured and predicted values (high accuracy of the model). Also, negative BIAS values indicate underfitting, while positive BIAS values reveal overfitted predictions (Steidle Neto *et al.*, 2016). The coefficient of determination (Eq. 5) represents the proportion of explained variance of the response variable in the validation set, with results varying from 0 to 1, and the maximum value reflecting a perfect agreement between measured and predicted data (Steidle Neto *et al.*, 2017). Finally, index of agreement (Eq. 6) varies from 0 to 1, where the maximum value reflects a perfect agreement between measured and predicted data. As affirmed by Willmott (1981), this is an important index since it is not a measure of correlation or association in the formal sense but rather a measure of the degree to which the model's predictions are error free.

$$MAE = \frac{\sum |Y_p - Y_o|}{n} \quad (3)$$

$$BIAS = \frac{\sum (Y_o - Y_p)}{n} \quad (4)$$

$$R^2 = \frac{[\sum (Y_p - \bar{Y}_p)(Y_o - \bar{Y}_o)]^2}{\sum (Y_p - \bar{Y}_p)^2 \sum (Y_o - \bar{Y}_o)^2} \quad (5)$$

$$d = 1 - \frac{\sum (Y_p - Y_o)^2}{\sum (|Y_p - \bar{Y}_o| + |Y_o - \bar{Y}_o|)^2} \quad (6)$$

Where MAE is mean absolute error (mg kg^{-1}), BIAS is the mean bias error (mg kg^{-1}), R^2 is the coefficient of determination (dimensionless), and d is index of agreement (dimensionless).

The limit of detection (LOD) and limit of quantitation (LOQ) are frequently used to describe the smallest concentrations of a sample that can be reliably measured by an analytical procedure. The LOD corresponds to the lowest analyte concentration at which detection is feasible, while the LOQ is the lowest concentration at which the analyte can be effectively quantified. Thus, LOQ tends to be equivalent or higher than LOD (Armbruster and Pry, 2008). In this study, the statistical LOD and LOQ determinations were applied (CLSI, 2004; Jeon *et al.*, 2007). That is,

the spectra of five representative blank lettuce samples (containing no dithiocarbamate residues) were measured, following the same procedures used during the calibration and external validation of the PLS model. The mean and standard deviation of the predicted CS_2 concentrations were calculated based on these spectra, which were used as input for the developed model. The LOD and LOQ were equal to the mean of the predicted CS_2 concentrations plus three times or ten times the standard deviation of the mean, respectively. According to CLSI (2004) and Armbruster and Pry (2008), although the blank samples are devoid of analyte, they can produce an analytical signal that might be consistent with a low concentration of dithiocarbamate.

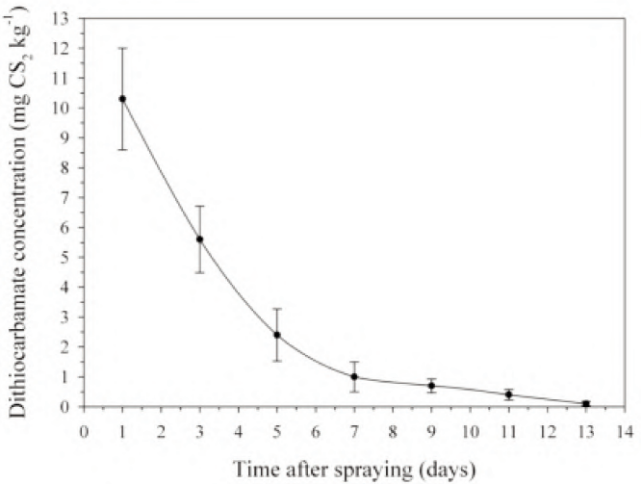

Fig. 2 - Dithiocarbamate concentration decay (mg CS_2 kg^{-1}) on lettuce leaves at seven intervals after mancozeb spraying. Vertical bars represent the standard error of the average values.

3. Results

Figure 2 presents the degradation curve of dithiocarbamate residues on lettuce leaves during the preharvest interval, obtained from average values of laboratory analytical measurements. The estimated half-life of the dithiocarbamate residues was 5-7 days. The rates of decline of dithiocarbamate concentration in two-day intervals were quite variable from first to seventh day after spraying (4.7, 3.2, and 1.4 mg CS_2 kg^{-1}), corresponding to a decrease of 90.3%. After the seventh day, the rates of decline were 0.3 mg CS_2 kg^{-1}, indicating a decrease of 8.7%. Although very small concentration residues persisted at the final of preharvest interval, the results indicate that after this period the pesticide metabolization is advanced, assuring reliability for lettuce consumption.

The PLS model for dithiocarbamate concentration on lettuces was more precise and accurate when detrending pre-treatment was applied to spectral signatures, compared with predictions obtained from spectra without pre-treatment or treated with other methods (centering, standardization, first and second derivatives). Despite NIR bands were more sensible, all wavelengths (350-1000 nm) presented potential to explain the dithiocarbamate concentration residues on lettuce leaves from spectral reflectance, contributing to the good performance of the prediction PLS model.

Table 1 shows the statistical results for the dithiocarbamate concentration model, considering the calibration with cross-validation and external validation datasets. The data processing showed that the use of more than four latent variables for dithiocarbamate concentrations resulted in an over-fitting, characterized by a slight divergence in the downward trend of the RMSE, which continued to decrease for calibration (RMSEC), but almost established for cross-validation (RMSECV).

The proposed PLS model was satisfactory since presented low RMSEC, RMSECV, RMSE, MAE, and BIAS when compared to the range values (Table 1). Additionally, the performance of the model for the external validation presented high index of agreement (0.94), reflecting a good accuracy for independent predictions of the dithiocarbamate concentra-

Table 1 - Statistical parameters of calibration with cross-validation and external validation processes for the estimation model of dithiocarbamate concentration on lettuces

	Calibration		External validation
Number of latent variables	4	R^2 (dimensionless)	0.87
RMSEC (mg CS_2 kg^{-1})	1.86	RMSE (mg CS_2 kg^{-1})	1.41
RMSECV (mg CS_2 kg^{-1})	2.74	MAE (mg CS_2 kg^{-1})	1.24
Range (mg CS_2 kg^{-1})	0.23-10.3	BIAS (mg CS_2 kg^{-1})	-0.37
		d (dimensionless)	0.94

tions on lettuces. The negative BIAS value indicated a majority tendency of underfitting predictions by the model, mainly from 2.3 mg CS_2 kg^{-1} (Fig. 3). Also, low BIAS value represented small systematic error (Table 1).

Regarding the differences among statistics presented in Table 1, the spectral measurements and predictions of external validation may deviate from the calibration as they originate from different sample sets. As mentioned by Liu and Ying (2005), in this way, the ability of the calibration model to withstand unknown variability is assessed.

The correlations between values determined by the reference analytical method and those predicted by the external validation of the PLS model are presented in figure 3. Prediction performance resulted in good agreement between reference and estimated values, with R^2 of 0.87 (Table 1), indicating that 87% of the measured values were accurately represented by the model.

The comparison of parameters (LOD, LOQ, and range) associated to different methods of determination of dithiocarbamates on lettuces are showed in Table 2. The LOD and LOQ values verified in this study were higher than those obtained with the gas or liquid chromatography, as well as the spectrophotometric method.

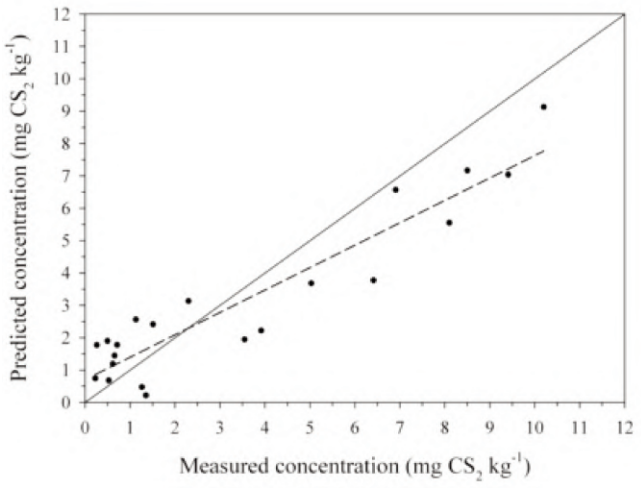

Fig. 3 - External validation of the PLS model for estimating dithiocarbamate concentrations (mg CS_2 kg^{-1}) on lettuce leaves.

The usefulness of the methodology presented in this study has been evidenced by the determination of dithiocarbamate residues on lettuce samples at concentrations between 0.23 and 10.3 mg CS_2 kg^{-1}. This range can be considered appropriated when the results of previous studies are used as reference (Table 2). Further, values between these lower and upper limits are sufficient to measure a wide range of dithiocarbamate concentrations on lettuces.

4. Discussion and Conclusions

Past studies showed dithiocarbamate degradation profiles for lettuces similar to those found in this study. Yip *et al.* (1971) found that the dithiocarbamate concentration (maneb) from a single spray on lettuces declined from 45 mg kg^{-1} initially to about 5 mg kg^{-1} after 15 days (decrease of 89%). On the other hand, Hughes and Tate (1982) monitored mancozeb residues on lettuces and verified a reduction of 90 mg kg^{-1} after a 14-day interval (decrease of 72%). These authors also reported a high initial rate of decline in dithiocarbamate concentration during the first seven days after spraying, confirming the high degradation of analyte.

Despite of the dithiocarbamates with active ingredient based on mancozeb are not authorized by ANVISA for lettuces in Brazil, the European Food Safety Authority (EFSA) allows the use of this pesticide on lettuce crop in the countries that integrate the European Union, considering a maximum residue limit of 5 mg kg^{-1} (EFSA, 2013). In addition to the dietary risk, the edafoclimatic differences and dietary patterns of the countries justify the distinct positions between ANVISA and EFSA. Based on the results, lettuces presented dithiocarbamate concentrations

Table 2 - Limit of detection, limit of quantitation, and range (expressed in mg CS_2 kg^{-1}) for different methods of determination of dithiocarbamates on lettuces

Method	Limit of detection	Limit of quantitation	Range	Reference
Gas chromatography	0.004	0.013	0.04-5.0	Česnik and Gregorčič (2006)
Liquid chromatography	0.04	0.11	0.50-9.3	López-Fernández *et al.* (2012)
Gas chromatography	0.02	0.05	0.04-1.0	Pizzutti *et al.* (2017)
Spectrophotometric	0.28	0.40	0.20-4.5	Pizzutti *et al.* (2017)
Spectrometric	0.49	1.41	0.23-10.3	Present study

lower than the maximum residue limit established by EFSA from the fourth day after spraying.

According to Lopes and Steidle Neto (2018) detrending, which was the most appropriated spectral pre-treatment in this study, is usually used to remove specific data offsets that are not related to the chemical or physical properties of interest for the chemometric modeling. Sánchez *et al.* (2010) also developed PLS models for predicting pesticide residues on intact peppers using near-infrared reflectance spectroscopy, applying detrending method for scatter correction in data, and obtaining good results. Steidle Neto *et al.* (2016) affirmed that detrending was the best spectra pre-treatment when predicting chlorophyll content in lettuces, helping to remove non-linear trends in spectroscopic data, and consequently correcting scatter.

The number of latent variables considered adequate for predicting dithiocarbamate concentrations on lettuce leaves in this study agree with Cozzolino *et al.* (2011), who affirmed that if more than optimum number of latent variables is used, the solution can become over-fitted and the model will be very dependent on the dataset, giving poor predictions. Otherwise, using less than the optimum number of latent variables will cause under-fitting and the model will not be accurate enough to capture the variability in the data. This result also agrees with other researchers who applied spectroscopy and PLS models to non-destructively predict of pesticide concentrations. Jamshidi *et al.* (2016) showed that 5-7 latent variables were required for PLS models when predicting diazinon residues on cucumbers.

Although the proposed model tended to underestimate the pesticide concentrations, slight overestimates were verified for low dithiocarbamate concentrations, evidenced in this study until the fifth day after spraying.

The method based on spectral reflectance provided sufficient sensibility for detecting and quantifying concentrations lower than the maximum residue limit allowed by the European Food Safety Authority for mancozeb-based dithiocarbamates on lettuces (5 mg kg^{-1}). Makino *et al.* (2009) used spectral reflectance for detection and quantification of chlorpyrifos residues on apple surface, also demonstrating the feasibility of spectroscopy for estimating low concentrations of pesticide residues in fruits.

According to Acharya *et al.* (2012), the spectral features can be assigned to overtone and combination bands of various C-H and N-H bonds within these molecules. Considering that the chemical structure of mancozeb has 2 NH and 2 CH_2, and that the NH bond position is unhindered within the chemical structure, there is a high likelihood it will produce a sharp and strong absorption band, which should improve detection of mancozeb.

The mean spectral signatures of intact lettuce leaves with absence and presence of fungicide presented differences mainly in NIR region, with fungicide-contaminated samples resulting in greater absorbance than that in the fungicide-free lettuce leaves. According to that reported by Sánchez *et al.* (2010) and Jamshidi *et al*. (2016), increase of the absorbance in NIR region after 900 nm could be due to the C-H absorption.

Based on the results, it can be said that the Vis/NIR spectroscopy combined to the multivariate data analysis have potential to be applied as an alternative method to estimate dithiocarbamate concentrations on lettuce leaves. However, the success and widespread adoption of this method also depends of suitable measurement practices. It is important that measurements are performed using a spectrometer with high spectral resolution, after the time required to warm-up the light source, and after adequate spectrometer calibration (proper sampling of reference standard). Additionally, important factors to achieve good results include the standardization of the measurement points in the samples, the homogeneity of the target area, and the positioning of samples on a black non-reflective panel with the purpose of prevent the light reflection going through the leaf. Another essential aspect is related to the incidence angle of the light bunch, which is emitted by the light source over the sample and directly influences the light reflection by sample (Steidle Neto *et al.*, 2017). Although the effects of the abovementioned individual error sources may appear small, their combined presence may result in measurements with significant errors, influencing the spectral reflectance independently of dithiocarbamate concentrations, as well as, the model predictions.

Vis/NIR spectral reflectance measurements combined to the partial least square analysis showed to be feasible and effective as a promising method for estimating concentration of dithiocarbamate fungicide residues on intact lettuce leaves. The developed PLS model allowed the detection and quantification of the dithiocarbamate without any preparation and/or processing of lettuce samples. This method offers advantages over traditional laboratory methods, such as real-time measurements and the possibility to be built into the industrial processing lines,

enabling large-scale individual analysis in 100% of the lettuces.

The spectral behavior of other vegetable species certainly will differ from that of lettuce, as well as, different dithiocarbamate types (thiram, metiram, propineb, zineb, ziram, and maneb) tend to cause variations in the estimating models. Future researches will be performed in order of evaluating these effects when detecting and quantifying fungicide residues in vegetable crops. The results of these new studies will complement the findings of the present work, making this a more wide-ranging method.

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Short note

Extraction of total protein from shoots of *Cereus* morphological variants (Cactaceae) for proteomic analysis

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Key words: cactus, phenotypic variants, protein extraction, SDS-PAGE, succulent tissues.

Abstract: Since there is a hypothesis that qualitative and/or quantitative differences of specific proteins may be associated with morphological variants in cacti of the *Cereus* genus (phenotypes erect, *tortuosus* and *monstruosus*), in current study we tested three different methods for protein extraction from shoots of the phenotypic variants to obtain protein fractions for further proteomic analysis. The TCA/acetone method for protein extraction revealed a larger number of well-defined bands in SDS-PAGE system than the methods with phenol. The quantification of protein extracted by TCA/acetone ranged between 0.488 (*tortuosus*) and 2.92 µg·mL^{-1} (*monstruosus*). Although the use of phenol is the most appropriate procedure for protein extraction from recalcitrant tissues, results have shown that extraction buffer containing two antioxidant agents (EDTA and β-mercaptoethanol) and PMSF to prevent protein degradation was efficient to avoid proteolysis and lower protein yield, than using TCA/acetone precipitation for protein extraction from shoots of *Cereus* sp. The use of extraction buffer with appropriate combinations of antioxidant agents, phenol-complexing agents, and protease inhibitors may be an efficient alternative for proteins extraction from succulent and recalcitrant tissues (such as cactus plants) using a simple protein extraction method.

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1. Introduction

Since the 1960s the study on proteins from plant tissues is frequently restricted by extraction procedures from different tissues (Loomis, 1969; 1974; Kelley and Adams, 1977). Different procedures for protein extraction have been reported for different tissues and plant species to obtain

protein fractions which are adequate for electrophoresis and polypeptides characterization on gel. Taxa and tissues have their own set of endogenous tannins, phenols and phenoloxidases which interfere differently with protein stability. Protein extraction from cactus tissues is particularly an arduous process due to the abundance of complex heteropolysaccharides, coupled to endogenous tannins, phenols and phenoloxidases in the shoot tissues of adult plants. Heteropolysaccharides confer high viscosity to the extraction solution after shoot-tissue homogenization and hinder the solubilization of proteins.

Protein extraction has been a primordial stage for the studies on proteomics. The detection of proteins and protein variants found in cells of a given tissue under specific conditions (functional genomic) (Wilkins *et al*., 1996; Westermeier and Naven, 2002) requires adequate solubilization and stability of protein fractions. The first step should establish a procedure for protein extraction from shoots, or rather, for the proteome analysis in shoots of phenotypic variants of cactus from genus *Cereus*. The *Cereus* plants have been mainly used as forage for ruminants (Silva *et al*., 2011) and fruits and pulp are used in the preparation of cookies, pies, and pastries (Almeida *et al*., 2007). Several studies have revealed the importance of the *C. peruvianus* species as fruit crop (Mizrahi, 2014). While the *C. jamacaru* plants constitute a wild natural resource in the semiarid region of Northeastern Brazil, an industrial and economic importance has been attributed to the *C. peruvianus* species cultivated in the Southern region of Brazil (Alvarez *et al*., 1995; Barros and Nozaki, 2002). Medicinal importance is also attributed to *C. peruvianus* since arabinogalactan extracted from the gum has been indicated for the treatment of gastric ulcers (Tanaka *et al*., 2010). Phenotypic variants of cacti from the genus *Cereus*, tagged *tortuosus* and *monstruosus* varieties, are frequently cultivated together with plants featuring typical columnar-erect shoots in home gardens and public parks and squares in Brazil´s southern region. Although the origin of the *tortuosus* and *monstruosus* varieties has not been reported in the literature, the qualitative and/or quantitative differences of specific proteins may be associated with the morphological variants.

Since the success of a proteomic experiment depend on the correct identification of proteins, three different methods described by He and Wang (2008) for protein extraction from shoots of the three phenotypic variants of *Cereus* were employed in the current study. The methods described by He and Wang (2008) were used in protein extraction from *Aloe vera* tissues, a succulent and recalcitrant plant similar to cacti. It is expected that protocols described by these authors may be suitable for protein extraction and quantification from shoots of *Cereus* plants.

2. Materials and Methods

Samples of *Cereus* plants with typically erect shoots and plants of the varieties *tortuosus* and *monstruosus* (Fig. 1), cultivated in south Brazil (in Maringá PR Brazil, at 23°25'38" S; 51°56'15" W), were collected from home gardens and maintained in the Experimental Botanic Garden of the State University of Maringá (altitude 554.9 m; 23°25'S; 51°25'W). Pieces of shoots (2 g) from erect, *tortuosus* and *monstruosus* plants (four plants of each morphology) were collected and used as samples for protein extraction. The cuticle and the fractions of cells with chlorophyll were removed from the pieces of shoot to minimize the contamination of the samples by polysaccharides.

Fresh shoot sections (2 g) of each *Cereus* plant with erect shoots (E1-E4) and plants of the varieties *tortuosus* (T1-T4) and *monstruosus* (M1-M4) were ground to a fine powder in liquid nitrogen and homogenized in 2 mL buffer 0.02 M Tris-HCl pH 7.5, 0.25 M sucrose, 0.01M ethylene glycol tetraacetic acid (EDTA), 0.001 M phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and 2% ß-mercaptoethanol, following method by He and Wang (2008). Proteins were extracted from two samples of each E1-E4, T1-

Fig. 1 - Different morphologies of *Cereus peruvianus* plants showing stems: erect (A), *tortuosus* (B), *monstruosus* (C).

T4, and M1-M4 plants (Table 1).

After homogenization, 1.0-mL aliquots from each *Cereus* plants were transferred to 2 mL microtubes and prepared by three procedures: i) TCA-acetone precipitation; ii) phenol extraction, and iii) improved phenol extraction method according to described by He and Wang (2008).

Protein was quantified by the fluorometric method in a Fluorometer Qubit® 1.0 using Qubit® Protein Assay kit from Life Technologies. Polyacrylamide gel (12%) was prepared with 16.2 mL of 30% acrylamide and 0.8% bis-acrylamide dissolved in 4 mL of 1.5 M Tris-HCl, pH 8.8, 107 µL 10% SDS, 5.7 mL twice-distilled water, 320 µL 2% ammonium persulfate and 16 µL TEMED. Stack gel was prepared at a final concentration of 5%, pH 6.8: 3 mL of 10% acrylamide and 5% bis-acrylamide dissolved in 3 mL of 0.24 M Tris-HCl pH 6.8, 30 µL twice-distilled water, 250 µL 2% ammonium persulfate and 3 µL TEMED. Further, 25 mM Tris/200 mM glycine, pH 8.3, and 0.1% SDS were employed in the electrode chambers.

Samples were taken from the freezer and 2 µL loading dye [20% glycerol 10 mM, Tris-HCl 1.5 M pH 8.8, 10% ($w \cdot v^{-1}$) bromophenol blue, 10% SDS ($w \cdot v^{-1}$), β-mercaptoethanol 2% ($v \cdot v^{-1}$), and twice-distilled water q.s.p.] were added to 8 µL of sample and applied to the gel. Electrophoresis was performed in Tris-glycine buffer at 200 volts, for thirty minutes, for the stacking gel, followed by two hours and thirty minutes for the separation of the proteins. After electrophoresis, the gel was fixed in a fixation solution (40% methanol; 70% acetic acid) and stained in silver 20% following protocol by Laemmli (1970). After migration, gels were stained, photographed and the images were analyzed with GelAnalyzer 19.1 software (http://www.gelanalyzer.com/) to transform electrophoretic bands into peaks, to calculate Rf and estimate the molecular weight of proteins.

The protein quantification ($\mu g \cdot \mu L^{-1}$) by the fluorometric method in duplicate of morphological variants of *Cereus* with shoots erect (E1-E4), *tortuosus* (T1-T4), and *monstruosus* (M1-M4) obtained by extraction with TCA/acetone method described by He and Wang (2008) were analyzed using the software R (R Core Team, 2019) with the packages: i) "nortest" (Gross and Ligges, 2015) to verify the normality of the errors by the Lilliefors test (Lilliefors, 1969), ii) "car" (Fox and Weisberg, 2019) to verify the independence of errors by the Durbin-Watson test (Durbin and Watson, 1951), and iii) the "ExpDes" package (Ferreira *et al*., 2018) to verify the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000). All these procedures were admitting the error at 1% of significance.

3. Results and Discussion

The TCA/acetone method for protein extraction from shoots of *Cereus* plants revealed a larger number of well-defined bands in SDS-PAGE system than the methods with phenol and improved-phenol extraction. In the TCA/acetone method, each homogenized tissue (plant tissues with shoots erect, *tortuosus* and *monstruosus*) in the extraction buffer was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was then recovered and placed in a new tube where ¼ of the volume of acetone containing 50% trichloroacetic acid (TCA) was added. The solution was incubated for 2 h, at -20°C and centrifuged again at 15,000 rpm for 40 min, at 4°C. The supernatant was then discarded and the pellet was

Table 1 - Protein quantification ($\mu g\ \mu L^{-1}$) by the fluorometric method in duplicate of morphological variants of *Cereus* with shoots erect (E1-E4), *monstruosus* (M1-M4), and *tortuosus* (T1-T4) obtained by the extraction with TCA/acetone method described by He and Wang (2008)

Erect		*Monstruosus*		*Tortuosus*	
sample	$\mu g \cdot \mu L^{-1}$	sam	$\mu g \cdot \mu L^{-1}$	sample	$\mu g \cdot \mu L^{-1}$
E1	0.824	M1	2.64	T1	1.090
	0.658		2.68		0.990
E2	0.732	M2	1.71	T2	0.768
	0.766		1.91		1.150
E3	0.964	M3	2.26	T3	1.740
	0.874		2.40		1.520
E4	1.100	M4	2.54	T4	0.488
	1.480		2.92		0.592

washed three times with acetone containing 0.2% 1,4-Dithiothreitol (DTT). Pellets were then dissolved in 50 µL lysis buffer (8 M urea, 4% NP-40 and 1% DTT) and stocked at -20°C.

The quantification of protein extracted by TCA/acetone method was made in duplicate (two samples of each shoot morphology: erect, *tortuosus* and *monstruosus*) and ranged between 0.488 (*tortuosus*) and 2.92 µg·mL^{-1} (*monstruosus*) (Table 1).

The normality of the errors by the Lilliefors test (Lilliefors, 1969), the independence of errors by the Durbin-Watson test (Durbin and Watson, 1951) and the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000) showed that there were no restrictions by *p*-values regarding analysis of variance (ANOVA) procedure (Table 2).

Significant differences (at 1% level) in protein concentrations within and between *Cereus* plants with the erect, *tortuosus* and *monstruosus* morphologies were detected by ANOVA procedure (Table 3).

The non-significant experimental error indicates that the sampling error (different morphologies) may justify the differences in protein concentrations between *Cereus* plant morphologies. Non-significant experimental error assures the researcher more reliable results because the effect of uncertainty is smaller (Patterson, 1946; Cochran and Cox, 1957; Snedecor and Cochran, 1980).

Table 2 - p-values of the normality of errors by the Lilliefors test (Lilliefors, 1969), independence of errors by the Durbin-Watson test (Durbin and Watson, 1951) and the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000)

Procedure	Normality	Independence	Variance
Lilliefors	0.8897		
Durbin-Watson		0.0379	
O´Neill and Matthews			0.4089

Table 3 - Mean Squares (MS) of the comparison of protein concentrations from the three *Cereus* morphologies (erect, *tortuosus* and *monstruosus* shoots) and their respective sampling and experimental mistakes

	MS
Morphologies	5.2477 **
Sample error	0.2969 **
Experimental error	0.0251

** *p*-value <0.01.

A higher protein concentration in *monstruosus* plants than in erect and *tortuosus* plants has been detected by Tukey´s test (Tukey, 1953) employed to compare the averages of protein concentrations in erect, *tortuosus* and *monstruosus* plants (Table 4). Figure 2 also illustrates the highest protein concentration in *monstruosus* plants and shows the amplitude of concentration rates within each plant.

Differences in protein concentrations in *Cereus* plants with the erect, *tortuosus* and *monstruosus* morphologies indicate that it is necessary to evaluate the protein concentration in more than one plant with the same morphology to specify the average protein concentration of each morphology. Differences in protein concentrations among *Cereus* plants with erect, *tortuosus* and *monstruosus* morphologies detected in current study support the hypothesis that qualitative and/or quantitative differences of specific proteins may be associated with morphological variants in cacti of the *Cereus* genus, and may be used to justify a proposal for further proteomic analysis.

The phenol extraction method described by He and Wang (2008) revealed a number of protein frac-

Table 4 - Mean protein concentrations (µg µL^{-1}) from the three *Cereus* morphologies (erect, *tortuosus* and *monstruosus*) revealed by the Tukey´s test

Morphology	Mean protein concentraction (µg·µL-1)
Monstruosus	2.3825 a
Tortuosus	1.0423 b
Erect	0.9248 b

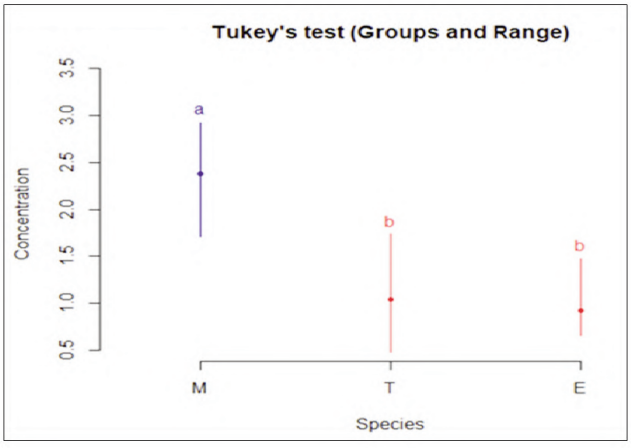

Fig. 2 - Amplitude of the concentration values observed within *monstruosus* (M), *tortuosus* (T), and erect (E) plants of *Cereus*.

tions smaller than the number of protein fractions detected by the TCA/acetone precipitation method in *Cereus* plants, while protein fractions were absent with the improved phenol extraction method described by He and Wang (2008)(Fig. 3). The GelAnalyzer software was useful to create calibration curves and to estimate the quantity of the protein from a band. Figure 4 shows the calibration curve that make a correspondence between the quantity of the protein loaded on each lane and the areas of the peaks of each lane (the software name the area of the peak as raw volume and the conventional units are in pixels). The correlation coefficients of such calibration curves were higher than 0.99 for proteins with molecular masses ranging from 24 until 180 kDa (Table 5). However, data obtained by MS (Mass Spectrometry analysis) indicated proteins with molecular weights varying from 220 to 15 kDa. In gel regions without clear band distinction in 1DE SDS-PAGE were detected considerable amounts of protein identified by MS. Preliminary Mass Spectrometry analysis identified 753 proteins extracted by the TCA/acetone precipitation method in the *Cereus* plants (unpublished results).

Improved phenol extraction method was described as the most appropriate procedure for pro-

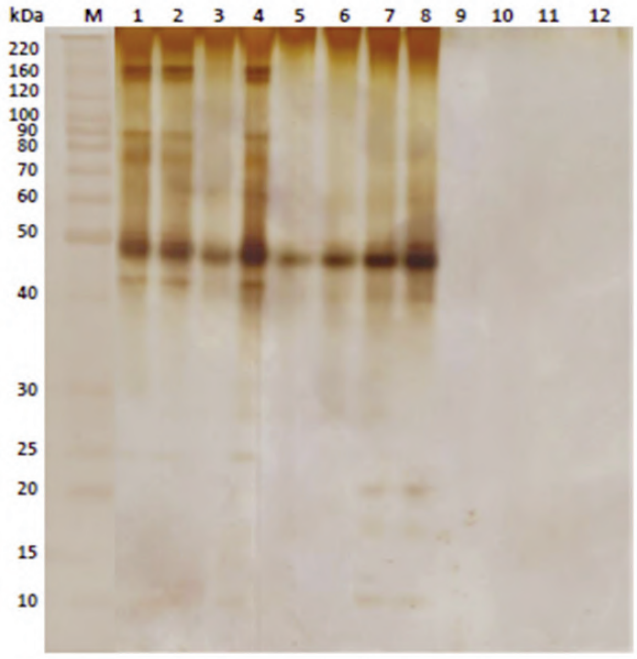

Fig. 3 - Protein extraction from shoots of *Cereus* with erect (samples 1-2, 5-6, 9-10), *tortuosus* (samples 3, 7, 11) and *monstruosus* (samples 4, 8, 12) morphologies, with TCA/acetone method (samples 1-4), with phenol extraction method (samples 5-8) and with the improved-phenol extraction method by He and Wang (2008) (samples 9-12), in the SDS-PAGE system 12%.

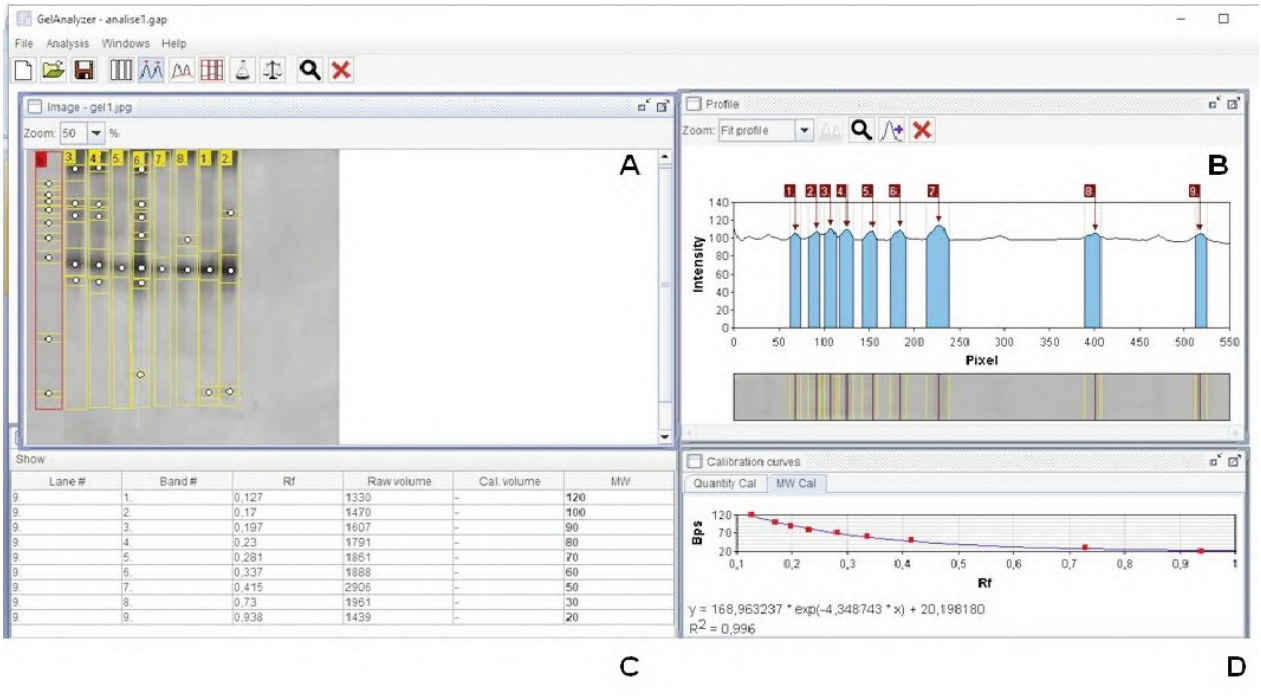

Fig. 4 - The main panels of Gel Analyser software showing the image of the entire electrophoresis gel (panel A); the bands from the selected lane from panel A are transformed in peaks (panel B) and their area are computed in panel C, as raw volume (conventional units). Based on the molecular weights of the markers (in panel D), the correspondence between the molecular mass and the migration (as Rf) of the protein is presented.

Table 5 - Quantity of the protein loaded on each lane detected with TCA/acetone method (samples 1-4) and with phenol extraction method (samples 5-8) in the SDS-PAGE system 12% from the *Cereus* plants

Lane	MW of each band (KDa)								
Lane 1	-	-	-	-	-	-	45	-	24
Lane 2	-	-	-	77	-	-	45	-	24
Lane 3	-	145	90	77	-	-	45	39	-
Lane 4	180	145	90	77	-	-	45	39	-
Lane 5	-	-	-	-	-	-	45	-	-
Lane 6	-	145	90	77	61	-	45	39	24
Lane7	-	-	-	-	-	-	45	-	-
Lane 8	-	-	-	-	-	58	45	-	-

tein extraction from *Aloe vera* tissues, a succulent plant, similar to cacti. Phenol has also been considered as the most effective for the removal of unwanted interfering substances from the protein samples of tissues from other recalcitrant plants (He and Wang, 2008; Pavoković *et al.*, 2012; Riffel *et al*., 2012). It has been postulated that, in the case of particularly recalcitrant tissues, acetone and TCA/acetone precipitation do not sufficiently remove nucleic acids, carbohydrates and polyphenols, which cause co-precipitation and degradation of proteins. The phenol method, although more laborious and time-consuming, resulted in higher protein yield and with the lowest contamination rate of samples than the TCA/acetone precipitation method alone for protein extraction from recalcitrant tissues (He and Wang, 2008; Pavoković *et al.*, 2012; Wu *et al.*, 2014).

However, results from the current study are indicative that the use of an extraction solution containing two antioxidant agents (EDTA and β-mercaptoethanol) and PMSF to prevent protein degradation was efficient to avoid proteolysis and lower yield of proteins using TCA/acetone precipitation for protein extraction from shoots of *Cereus* sp. Although the use of phenol is reported to be the most appropriate procedure for protein extraction from recalcitrant tissues, a significant proportion on cellular proteins may be lost from the extracts during the extraction procedure with phenol. The phenol extraction method involves more steps than the TCA/acetone method while a 'minimum-steps´ method is preferred with protein extraction procedures (Isaacson *et al*., 2006). The TCA/acetone method described by He and Wang (2008) involved only one precipitation, two centrifugations and three washes after homogenization of the tissues, whereas nine steps (including one overnight precipitation at -20°C) were reported for phenol method. Thus, the use of the TCA/acetone method described by He and Wang (2008) represented a minimum-steps method and, thus, a shorter time for the extraction of proteins from shoot tissues of *Cereus* plants.

A relatively simple extraction buffer containing one phenol-complexing agent (PVP) and two antioxidant agents (β-mercaptoethanol and EDTA) was also used for electrophoresis of the several isozymes in shoot tissues of the cactus *Cereus peruvianus* (Mangolin and Machado, 1997), while phenol and ammonium acetate were employed for the protein extraction from callus tissues of *C. peruvianus* (Mangolin *et al.*, 1999) for two-dimensional electrophoresis of callus tissues grown in culture media containing different concentrations of auxin and cytokinin. Only one phenol-complexing agent (PVP) and a cocktail of protease inhibitors (Roche, USA) were used for protein extraction from tissues of the cactus *Mammillaria gracilis* grown in vitro (Rogic´ *et al*., 2015). The TCA/acetone/β-mercaptoethanol method plus protease inhibitors (Roche, USA) was also employed for protein extraction from several species of succulent plants and some cactus species (*Lophocereus marginatus*, *Mammillaria magnimamma* and *Opuntia ficus-indica*) instead of phenol precipitation method (Lledías *et al*., 2017). The evidence from these studies actually demonstrates that, in the case of tissues with different levels of interfering substances, different phenol complexing agents and different antioxidant agents are required. The physiological state of each tissue is the decisive factor of a greater or lesser complexity in protein extraction processes. As types and concentrations of polysaccharides, polyphenols and other secondary metabolites may be induced in response to environmental factors, different types and concentrations of phenol-complexing agents and antioxidant agents may be needed for the same tissue in different environmental conditions. According to Wendel and Weeden (1989), extraction solutions for tissues with moderate

levels of interfering substances require at least two phenol-complexing and two antioxidant agents, while at least four phenol-complexing and three antioxidant agents are needed for tissues with high levels of interfering substances.

4. Conclusions

The simplest method (TCA/acetone precipitation), described by He and Wang (2008), and the use of two antioxidant agents and a protease inhibitor showed a number of protein fractions greater than the number of protein fractions detected with the phenol method in shoots of *Cereus* analyzed in current study (plants with erect shoots and plants of the varieties *tortuosus* and *monstruosus*). The authors´ expectation is to use this simple method of extracting proteins for proteomic analysis of the phenotypic variants from the genus *Cereus*. However, the use of phenol or other phenol-complexing and antioxidant agents to extract proteins from shoots of other *Cereus* plants grown in different regions or in different environmental conditions (different seasons of the year, e.g.) may be needed. Consequently, the proposal to establish a 'universal protocol' for succulent plants, such as cacti, seems unattainable. Therefore, testing with extraction buffers and precipitation methods, which may be time-consuming and laborious, seems indispensable for further analysis of other *Cereus* plants in a proteomic-based approach.

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