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

AHS

Advances in Horticultural Science

Vol. 30 - n. 1, 2016

FIRENZE UNIVERSITY PRESS

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

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

ADVANCES IN HORTICULTURAL SCIENCE

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

supported by ENTE CASSA DI RISPARMIO DI FIRENZE

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

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

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

CONTENTS

Adv. Hort. Sci., 2016 30(1): 3-11

Impact of drought on flowering, yield and quality parameters in diverse genotypes of tomato (*Solanum lycopersicum* L.)

R. Sivakumar (*), S. Srividhya
Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India.

Key words: ascorbic acid, drought, flower abscission, lycopene, soluble protein, SPS, yield.

Abstract: The effect of drought stress on flowering, yield and quality of tomato (*Solanum lycopersicum*) genotypes was investigated under field conditions in rainout shelter. The drought condition was imposed on the first day after transplanting based on field capacity of soil. Experimentation was undertaken with ten genotypes adopting Factorial Randomized Block Design with three replications and two treatments *viz.,* 1.0 IW/CPE and 0.5 IW/CPE field capacity. As the stress increased from 100% field capacity to 50% field capacity, reductions in chlorophyll index, soluble protein content, days to flower initiation, sucrose phosphate synthase (SPS) activity, fruit volume, fruit diameter, yield and increased flower abscission percentage were noted. Significant increases in TSS and lycopene were observed under drought. The genotypes LE 118, LE 57 and LE 114 showed significantly less reduction in soluble protein content; SPS activity and fruit yield during drought were considered as drought tolerant. Genotypes LE 1 and LE 125, which gave the lowest soluble protein content, SPS activity and ultimately poor yield, were considered as drought susceptible.

1. Introduction

Drought stress can affect plant growth, development and yield. It has been estimated that up to 45% of world agricultural lands are subjected to drought (Bot *et al*., 2000). Water deficit leads to the perturbation of most of the physiological and biochemical processes and consequently reduces plant growth and yield (Boutraa, 2010). Abscission of reproductive organs like flower buds and flowers is a major yield-limiting factor in vegetable crops (Wien *et al*., 1989). The abscission of floral organs during stresses has been associated with changes in physiological processes (Aloni *et al*., 1996).

It has been reported that, in tomato, the abscission of flowers and flower buds and the reduction in photosynthesis were higher in susceptible cultivars compared to tolerant cultivars (Bhatt *et al*., 2009). In soybean, flower retention and fruit set are highly sensitive to environmental stresses (Kokubun *et al*., 2001). Etsushi *et al*. (2009) reported that Soil Plant Analytical Development (SPAD) method readings significantly correlated with chlorophyll content, rubisco content, photosynthetic rate, and Fv/Fm ratio. A major portion of soluble protein (50%) in leaves is occupied by rubisco, a prime enzyme for carbon fixation in photosynthesis (Noggle and Fritz, 1986). Daniel and Triboi (2002) showed that heat stress decreased the duration of soluble protein accumulation in terms of days after anthesis but not in terms of thermal time.

Drought decreased leaf N, whereas heat stress did not influence it and, however, the total soluble protein content was decreased during drought, heat, and a combination of drought and heat. Heat and drought stress induced suppression of photosynthesis by mainly decreasing the proportion of soluble protein to total leaf N, adversely affecting the rubisco protein and activity (Xu and Zhou, 2006). Bhatt *et al*. (2009) reported there was a considerable decrease in SPS activity in flowers under water deficit condition; a relatively higher decrease was observed in the susceptible genotypes. SPS activity was shown to decrease during leaf desiccation (Foyer *et al*., 1998), or to remain constant (Zrenner and Stitt, 1991). However, Yang *et al*. (2002) and Niedzwiedz-Siegen *et al*. (2004) observed an increase in SPS activity in rice and wheat leaves, respectively, under

(*) Corresponding author: sivatnau5@gmail.com
Received for publication 26 January 2015
Accepted for publication 6 November 2015

mild drought.

Tomato (*Solanum lycopersicum*) is one of the most popular and widely grown vegetables in the world. Considering the potentiality of this crop, there is plenty of scope for its improvement, especially under the drought situation. Water is a scarce resource for irrigation. Although the concept of drought tolerance has been viewed differently by molecular biologists, biochemists, physiologists and agronomists, the major concern is to enhance the biomass and yield under limited water input, which is a characteristic feature of rainfed agriculture.

There are several physiological and biochemical traits contributing to the drought tolerance of horticultural crops. However, a large number of tomato genotypes have not been screened for drought tolerance or exploited for their cultivation under drought. To breed drought tolerant genotypes, it is necessary to identify physiological traits of plants which contribute to drought tolerance. Chlorophyll pigments and soluble protein content in leaves which have a greater correlation to crop yield show reduced levels under drought. The reproductive stage of the crop is highly sensitive to any abiotic stress. Hence, reduction in crop yield under drought is mainly attributed to the effect on flowering characters and a decrease in SPS enzyme activity. Therefore, the present investigation was carried out to study the physiological traits such as SPAD value, soluble protein content, SPS activity, flowering characters, and yield to facilitate the screening and selection of tomato genotypes for drought tolerance.

2. Materials and Methods

Plant materials and cultivation

The study was undertaken to determine the effect of drought on flowering and yield of tomato genotypes in the field experiment at Rainout Shelter of Crop Physiology Department, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu during 2012-13. The experiment was conducted with 10 tomato genotypes (LE 1, LE 27, LE 57, LE 114, LE 118, LE 125, CO 3, PKM 1, TNAU THCO 3 and COTH 2) and two treatments (1.0 and 0.5 IW/CPE (Irrigation Water/ Cumulative Pan Evaporation) with three replications following a Factorial Randomized Block Design. Seeds of selected genotypes were sown in trays filled with vermicompost for nursery use. Twenty-five day old seedlings were transplanted in the rainout shelter; plot size 1.35 x 1.5 m. Drought was imposed on the first day after transplanting to all genotypes in both IW/CPE ratio treatments. Furrow irrigation was applied when the cumulative pan evaporation reading reached 50 mm (1.0 IW/CPE ratio) and 75 mm (0.50 IW/CPE ratio). Crop was supplied with FYM (25 t/ha rate), NPK fertilizers (75:100:50 kg/ha), borax 10 kg and zinc sulphate 50 kg/ha as basal dose and 75 kg N/ha on the 30th day after planting during earthing up. Other cultivation operations including weed control (Applied Pendimethalin 1.0 kg a.i./ha as pre-emergent herbicide followed by one hand-weeding at 30 days after planting) and plant protection measures were carried out as per the recommended package of practices of Tamil Nadu Agricultural University, Coimbatore.

Physiological parameters

SPAD readings were recorded using Chlorophyll Meter (SPAD 502) designed by the Soil Plant Analytical Development (SPAD) section, Minolta, Japan. Data were recorded as described by Peng *et al*. (1996). Soluble protein content of the leaf was estimated as per the method of Lowry *et al*. (1951) and expressed as mg g^{-1} FW. Sucrose phosphate synthase (SPS) activity was determined as described by Pavlinova *et al*. (2002). Sucrose was measured using anthrone reagent as modified by Ashwell (1957). Absorbance was measured at 630 nm and the activity was expressed in mg sucrose mg^{-1} protein h^{-1}.

Flowering parameters

Days to flowering (the number of days from seedling emergence to opening of the first flower) were recorded for each plant in the three replications and the average was taken. Abscission study was conducted on single flower basis. Flower number of tagged plants and dropped flowers per plant were counted every three days. These records were used to calculate the flower abscission and expressed in terms of percentage.

Yield parameters

Average fruit weight was calculated by adding the weight of five fruits from each plant at second harvest and dividing it by the total number of fruits and expressed in g $fruit^{-1}$. The fruit volume was estimated by water displacement method. Individual fruits were immersed in 1 L of water; amount of water displaced was measured and volume was worked out per fruit and expressed as cc. Polar diameter was measured from stalk end to blossom end of fruit by using Vernier Calipers and the average of five fruits

was worked out and expressed in cm. The fruit weight per plant was recorded in control and stressed plants for each picking and fruit yield (kg per plant) was calculated as fresh weight of fruits in all the pickings.

Quality parameters

Juice extracted from cut fruit was used to determine TSS with the help of a Hand Refractometer (0 to 32°Brix) at room temperature and the value was noted in °Brix. Ripened fruit samples were analyzed for ascorbic acid content, using 2,6-Dichlorophenol indophenol dye titrimetrically as per Sadasivam and Manickam (1996). Lycopene content of fruit was extracted using petroleum ether and OD of the extract was measured at 503 nm in UV-VIS-spectrophotometer using petroleum ether as a blank (Ranganna, 1986). Lycopene content of the sample was calculated using the following formula and expressed in mg 100 g^{-1}.

$$\text{Lycopene} = \frac{3.1206 \times \text{OD of sample} \times \text{volume made up} \times \text{dilution}}{\text{Weight of sample} \times 1000} \times 100$$

Statistical analysis

Data from the various parameters were analyzed statistically as per the procedure of Gomez and Gomez (1984).

3. Results

Decreased SPAD value under drought

Control (1.0 IW/CPE) plants showed a higher mean chlorophyll index value (47.57) than treated (0.5 IW/CPE) plants (44.04). Among the genotypes, CO 3 and PKM 1 recorded significantly higher chlorophyll index values of 50.1 and 46.1, and 49.5 and 46.7 at 1.0 IW/CPE and 0.5 IW/CPE conditions, respectively. During drought, the genotypes LE 114 (45.8), LE 57 (45.7), COTH 2 (45.5) and LE 118 (45.0) were found to be on par with each other.

Decreased soluble protein due to drought

Control (1.0 IW/CPE ratio) plants showed a higher mean soluble protein content (13.26) than the drought (0.5 IW/CPE ratio) imposed plants (8.98). Among the genotypes, COTH 2 (15.63) and THCO 3 (15.18) registered the highest soluble protein content under 1.0 IW/CPE ratio level and LE 57 (11.99) and LE 118 (11.74) under drought conditions (0.5 IW/CPE). The lowest soluble protein content was found for LE 125 (8.16) and LE 1 (8.68) (Table 1).

Table 1 - Effect of drought on chlorophyll index SPAD value and soluble protein content of tomato genotypes at 60 DAT

Genotypes	SPAD value			Soluble protein (mg g^{-1})		
	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean
LE 1	42.00	38.40	40.20	10.85	6.51	8.68
LE 27	48.20	44.40	46.30	13.98	10.72	12.35
LE 57	47.60	45.70	46.70	15.03	**11.99**	13.51
LE 114	48.20	45.80	47.00	13.43	10.19	11.81
LE 118	48.40	45.00	46.70	14.58	**11.74**	13.16
LE 125	45.00	39.50	42.30	11.07	5.24	8.16
CO 3	50.10	46.10	48.10	11.55	8.69	10.12
PKM 1	49.50	**46.70**	48.10	11.33	7.69	9.51
THCO 3	48.40	43.30	45.90	15.18	8.46	11.82
COTH 2	48.30	45.50	46.90	15.63	8.58	12.11
Mean	**47.57**	44.04	45.82	**13.26**	8.98	11.12
	G	**T**	**G x T**	**G**	**T**	**G x T**
SEd	1.15	0.38	1.62	0.137	0.061	0.194
CD (0.05)	2.28	0.76	NS	0.278	0.124	0.393

Altered flowering characters from drought

Our data on number of days to flower initiation revealed that genotypes, treatments and interactions attained statistical significance (Table 2). In the case of treatments, the plants under drought initiated flowers earlier (26) than control plants (30). At 0.5

Table 2 - Effect of drought on flowering characters of tomato genotypes during flowering stage

Genotypes	Days to flower initiation (DAT)			Flower abscission (%)		
	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean
LE 1	29	25	27	10.90	**20.10**	15.50
LE 27	30	27	29	13.20	15.80	14.50
LE 57	30	25	28	12.60	15.10	13.80
LE 114	29	26	28	11.90	15.40	13.70
LE 118	32	**28**	30	10.90	13.90	12.40
LE 125	31	25	28	11.90	17.10	14.50
CO 3	26	23	25	10.20	15.90	13.10
PKM 1	28	24	26	9.90	16.40	13.20
THCO 3	32	**28**	30	10.90	17.40	14.20
COTH 2	31	27	29	11.20	**18.10**	14.70
Mean	**30**	26	28	11.40	**16.50**	13.90
	G	**T**	**G x T**	**G**	**T**	**G x T**
SEd	0:18	0:08	0:26	0.08	0.03	0.11
CD (0.05)	0:37	0:17	0:52	0.16	0.07	0.22

IW/CPE ratio level, LE 118, THCO 3 (28), LE 27 and COTH 2 (27) registered a delay in flowering compared to other genotypes. The variety CO 3 showed its supremacy for earlier flowering, both under control (26) and drought (23) conditions, compared to the other genotypes; PKM 1 (28 and 24) ranked next.

The differences due to genotypes, treatments and interactions attained significance for flower abscission (Table 2). Among the treatments, the plants imposed with 1.0 IW/CPE ratio recorded lesser mean percentage of flower abscission (11.4) than 0.5 IW/CPE ratio plants (16.5). Among the genotypes, LE 1 recorded greater mean percentage of flower abscission (15.5) than the other genotypes considered. Interestingly, among the control plants, LE 27 and LE 57 recorded higher flower abscission (13.2, 12.6) while, under drought, LE 1 and COTH 2 recorded higher flower abscission of 20.1 and 18.1, respectively. At 0.5 IW/CPE ratio level, LE 118 showed its supremacy with lower abscission of 13.9 followed by LE 57 (15.1), LE 114 (15.4) and LE 27 (15.8).

SPS is the plant enzyme thought to play a major role in sucrose biosynthesis. It is considered to play a major role in the re-synthesis of sucrose (Wardlaw and Willenbrink, 1994) and sustain the assimilatory carbon flux from source to developing sink (Isopp *et al*., 2000). Among the genotypes, LE 57 recorded significantly higher enzyme activity of 2.75 under control followed by the genotypes LE 27 (2.61), LE 118 (2.53) and LE 114 (2.46). At 0.5 IW/CPE ratio level, the highest activity of 1.97 was registered by LE 118 followed by LE 57 (1.90) while the lowest was recorded by LE 1 (0.57) and LE 125 (0.69) (Fig. 1).

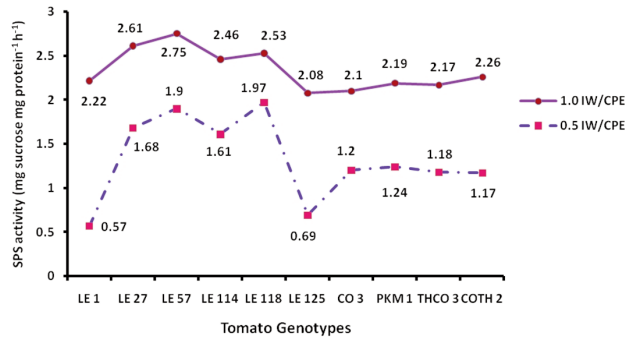

Fig. 1 - Effect of drought on SPS activity of tomato genotypes at 60 DAT.

The data on yield components such as average fruit weight, fruit volume, fruit diameter, fruit number and fruit yield attained statistical significance except fruit diameter. Comparing the treatments, control (1.0 IW/CPE ratio) plants recorded higher fruit weight (20.25) than under drought condition (13.75) (Table 3). Relating the genotypes, COTH 2 recorded higher fruit weight of 45.40 which was on par with THCO 3 (44.45) at 1.0 IW/CPE ratio level while the lowest was registered by LE 125 (9.06) followed by LE 57 (9.48). At 0.5 IW/CPE ratio level, relatively higher fruit weight was recorded by COTH 2 (29.87) followed by THCO 3 (28.78), CO 3 (19.93),

Table 3 - Effect of water deficit on the yield parameters of tomato genotypes

	Average fruit weight (g)			Fruit volume (cc)			Fruit diameter (cm)		
Genotypes	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean
LE 1	11.18	5.72	8.45	12.020	6.48	9.25	3.41	2.43	2.920
LE 27	10.68	7.23	8.96	11.52	7.95	9.74	3.34	3.05	3.20
LE 57	9.48	7.89	8.69	10.09	8.62	9.36	3.11	3.48	3.30
LE 114	9.55	7.49	8.52	10.31	8.25	9.28	3.53	3.25	3.39
LE 118	10.01	**7.95**	8.98	10.92	8.61	9.77	4.12	3.76	3.94
LE 125	9.06	3.89	6.48	9.65	4.64	7.15	3.30	2.72	3.01
CO 3	26.38	19.93	23.16	28.39	22.69	25.54	5.40	4.84	5.12
PKM 1	26.31	18.75	22.53	28.34	22.49	25.42	5.51	4.71	5.11
THCO 3	44.45	**28.78**	36.62	48.02	**29.64**	98.33	6.57	5.59	6.08
COTH 2	45.40	**29.87**	37.64	48.95	**30.69**	39.82	6.31	**5.73**	6.02
Mean	**20.25**	13.75	17.00	**21.82**	15.01	18.41	**4.46**	3.96	4.21
	G	**T**	**G x T**	**G**	**T**	**G x T**	**G**	**T**	**G x T**
SEd	0.582	0.260	0.823	0.594	0.266	0.841	0.053	0.024	0.075
CD (0.05)	1.178	0.527	1.667	1.203	0.538	1.702	0.108	0.048	0.152

PKM 1 (18.75), LE 118 (7.95) and LE 57 (7.89).

The data on fruit volume recorded similar trend of fruit weight. Regarding the treatments, the plants imposed with 1.0 IW/CPE ratio recorded the fruit volume of 21.82 than 0.5 IW/CPE ratio (15.01) (Table 3). Under control, higher volume of fruit was recorded in the genotype COTH 2 (48.95) which was on par with THCO 3 (48.02). Under 0.5 IW/CPE ratio level, the lowest fruit volume was recorded by LE 125 (4.64) followed by LE 1 (6.48). Other than hybrids and varieties, LE 57 showed higher fruit volume of 8.62 followed by LE 118 (8.61), LE 114 (8.25) and LE 27 (7.95) at 0.5 IW/CPE ratio level.

With regard to fruit diameter, THCO 3 (6.08) and COTH 2 (6.02) recorded higher average diameter of fruits which was on par with each other. For treatments, plants imposed with 1.0 IW/CPE ratio recorded higher fruit diameter (4.46) than 0.5 IW/CPE ratio (3.96) (Table 3). Among the genotypes, COTH 2 registered higher fruit diameter of 5.73 followed by THCO 3 (5.59), CO 3 (4.84) and PKM 1 (4.71) at 0.5 IW/CPE ratio level. Other than hybrids and varieties, LE 118 showed higher fruit diameter of 3.76 followed by LE 57 (3.48), LE 114 (3.25) and LE 27 (3.05) at 0.5 IW/CPE ratio condition while the lowest was recorded by LE 1 (2.43) and LE 125 (2.72).

Comparing the irrigation treatments, plants that received 1.0 IW/CPE ratio recorded higher fruit yield than 0.5 IW/CPE ratio (Fig. 2). Among the genotypes, LE 57 recorded significantly superior fruit yield of 16.64 followed by COTH 2 (15.89), LE 118 (15.02), THCO 3 (14.67) and LE 27 (14.42) with 1.0 IW/CPE ratio level. But, at 0.5 IW/CPE ratio condition, LE 57 documented higher fruit yield of 11.12 followed by LE 118 (10.14), LE 114 (8.54) and LE 27 (8.13) while the lowest yield of 2.22 was recorded by LE 125 followed by LE 1 (2.57).

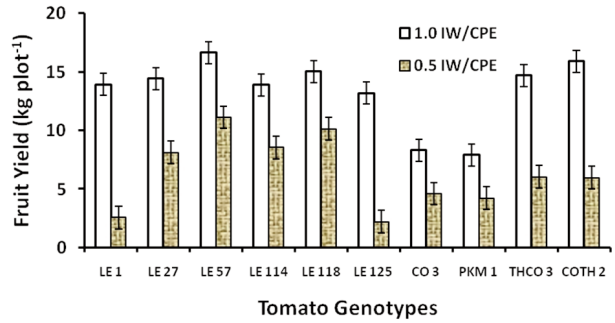

Fig. 2 - Effect of drought on the yield of tomato genotypes.

Quality parameters were altered under drought

The data on TSS content of the fruits revealed that the genotypes, treatments and interactions attained statistical significance (Table 4). Among the treatments, plants imposed with 0.5 IW/CPE ratio recorded higher brix value (3.01) than 1.0 IW/CPE ratio (2.89). Among the genotypes, THCO 3 recorded higher average brix value of 4.00 than the rest of the genotypes. At 0.5 IW/CPE ratio condition, the highest TSS value was recorded by THCO 3 (4.1) followed by COTH 2 (3.9), PKM 1 (3.6) and CO 3 (3.4) while the lowest was registered by LE 125 (2.2).

Plants imposed with 0.5 IW/CPE ratio recorded

Table 4 - Effect of water deficit on fruit quality of tomato genotypes

	TSS (° Brix)			Lycopene (mg 100 g^{-1})			Vitamin C (mg 100 g^{-1})		
Genotypes	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean	1.0 IW/CPE	0.5 IW/CPE	Mean
LE 1	2.50	2.70	2.60	2.21	2.39	2.30	14.45	14.42	14.44
LE 27	2.50	2.60	2.55	2.52	2.73	2.63	14.76	14.96	14.86
LE 57	2.40	2.60	2.50	2.46	2.68	2.57	14.95	15.30	15.13
LE 114	2.40	2.50	2.45	2.82	2.88	2.85	13.97	14.05	14.01
LE 118	2.40	2.50	2.45	2.85	2.95	2.90	14.36	14.36	14.36
LE 125	2.20	2.20	2.20	2.13	2.67	2.40	13.46	13.43	13.45
CO 3	3.30	3.40	3.35	4.54	**4.84**	4.69	24.05	**24.17**	24.11
PKM 1	3.50	3.60	3.55	3.78	4.05	3.92	23.06	23.21	23.14
THCO 3	3.90	**4.10**	4.00	3.35	3.53	3.44	15.42	15.64	15.53
COTH 2	3.80	**3.90**	3.85	3.54	3.55	3.55	16.19	16.30	16.25
Mean	**2.89**	**3.01**	2.95	3.02	**3.23**	3.12	16.47	**16.58**	16.53
	G	**T**	**G x T**	**G**	**T**	**G x T**	**G**	**T**	**G x T**
SEd	0.03	0.01	0.04	0.048	0.022	0.068	0.147	0.066	0.208
CD (0.05)	0.05	0.02	0.07	0.097	0.044	0.138	0.297	NS	NS

higher lycopene content (3.23) than 1.0 IW/CPE ratio (3.02). With respect to the genotypes, CO 3 recorded significantly higher average lycopene content (4.69). At 0.5 IW/CPE ratio level, lowest lycopene content was recorded by LE 1 (2.39) and LE 125 (2.67). The data on vitamin C content indicated that a narrow increment under drought compared to control (Table 4). 0.5 IW/CPE ratio recorded higher ascorbic acid (16.58) than 1.0 IW/CPE ratio (16.47). Among the genotypes, CO 3 recorded higher average vitamin content of 24.11 followed by PKM 1 (23.14). At 0.5 IW/CPE ratio level, the higher value was registered by CO 3 (24.17) followed by PKM 1 (23.21), COTH 2 (16.30) and THCO 3 (15.64) while the lowest values were recorded by the genotypes LE 125 (13.43) and LE 114 (14.05). Interestingly, only genotypes attained significant difference not the treatments or interactions in the case of ascorbic acid.

4. Discussion and Conclusions

In the present study, SPAD value, an index for total chlorophyll content in plants, showed a reduction under drought stress. Hawkins *et al.* (2009) reported that SPAD values can be used to evaluate the response of plant species to drought and heat stresses in the field. The adverse effect of drought on greenness of the leaf in the current investigation could be observed in the susceptible genotypes LE 125 and TNAU THCO 3 which depicted the highest reduction of SPAD values at the time of reproductive development stage. On the contrary, the tolerant genotype LE 57 showed only a very low reduction in SPAD value. Hence, the intensity of greenness in terms of SPAD values of the plant influenced the photosynthetic rate and thereby plant efficiency for increased biomass production. Ma *et al.* (1995) also reported a highly significant correlation of SPAD readings with photosynthetic rate in soybean.

The ability of the genotypes LE 57 and LE 114 to maintain high SPAD values under field conditions in response to water deficit has been revealed. Therefore, these genotypes were able to endure drought injury better than the sensitive lines.

The soluble protein content of the leaf, a measurement of RuBP carboxylase activity, was considered an index for photosynthetic efficiency. Rubisco enzyme makes up nearly 80% of the soluble proteins in leaves of many plants (Joseph *et al*., 1981). Diethelm and Shibles (1989) opinioned that, the Rubisco content per unit leaf area was positively correlated with that of soluble protein content of the leaf.

Several studies have reported that drought stress in tomato (Bartholomew *et al*., 1991), *Arabidopsis* (Williams *et al*., 1994), and rice (Vu *et al*., 1999) leads to a rapid decrease in the abundance of Rubisco small subunit (rbcS) transcripts, which may indicate the decreased synthesis of soluble protein.

The present study also confirms the above findings with a 32.3% reduction of soluble protein content under drought compared to control. Drought stress induces degradation of soluble proteins and this effect could be revealed through a reduction in leaf soluble protein content of various genotypes. In the present study, the reduction was, however, low in LE 118 and high in COTH 2 and THCO 3 under drought stress. Maintenance by the genotypes of soluble protein content could be attributed to higher Rubisco activity, leading to more carbon fixation and ultimately to higher photosynthetic efficiency under drought, which is one of the important traits for drought tolerance.

Reproduction is the crucial stage to be affected by any abiotic stress in any crop. An increase in the frequency of water stress days during flower development affects plant reproduction with immediate and long-term effects (Srivastava *et al*., 2012). Drought stress, in general, induces early flowering and in the present study as well flower initiation occurred three days earlier than the control. This early flowering under drought might be due to rapid phenological development in order to complete the life cycle under an unfavorable environmental condition.

Differences due to genotypes, treatments and interactions attained significance for flower abscission (Table 2). Among the treatments, the plants imposed with 1.0 IW/CPE ratio recorded a lower mean percentage of flower abscission than plants under 0.5 IW/CPE. Among the genotypes, LE 1 recorded the highest percentage of flower abscission. Interestingly, under control conditions, genotypes LE 27 and LE 57 gave high flower abscission, while LE 1 and COTH 2 recorded higher flower abscission under drought. An earlier finding by Bhatt *et al*. (2009) in tomato strongly supports the results of the present study.

A lower rate of flower abscission in the tolerant genotypes might be due to the maintenance of photosynthesis and efficient translocation of photosynthates to the reproductive parts under drought. The reduction in photosynthesis during stress may decrease the availability of assimilates to the devel-

oping floral organs and leads to the abscission of flowers and flower buds in susceptible cultivars. However, some workers are of the opinion that the abortion of reproductive organs is not solely due to a poor assimilate supply but also due to other factors such as assimilate utilization (Ruiz and Guardiola, 1994; Aloni *et al*., 1996).

In the present study, there was a reduction in SPS activity under drought conditions compared to control. The highest percent reduction (74.3) was observed in the genotype LE 1, however the lowest reduction was noted for LE 118. As observed also by Huber and Huber (1996), there was a significant elevation in SPS activity in response to water stress. In contrast to this finding, the present study revealed a decreased activity of SPS under drought conditions. Bhatt *et al*. (2009) observed a considerable reduction in SPS activity in susceptible cultivars during stress. The present study corroborates these findings. The reduced photosynthesis during water stress may also lead to a reduction in the capacity for both starch and sucrose synthesis and cause a decline in the SPS activity (Vassey and Sharkey, 1989).

Our results reveal that drought stress caused the reduction in fruit weight up to 30% under field conditions. Among the genotypes, LE 57, LE 118, and LE 114 had a lower reduction in fruit weight. All the genotypes exhibited a similar trend in fruit volume and fruit diameter in response to drought stress, which also caused remarkable changes in fruit number and an overall reduction up to 31% was observed compared to control. The reduction in fruit weight, in response to drought stress, had a direct influence on fruit yield of the various genotypes of tomato. Drought stress resulted in an overall yield loss of tomato fruits up to 55%. The greatest yield loss (70 to 80%) was exhibited by LE 1 and LE 125. The varieties and hybrids showed a reduction of fruit yield from 40.5 to 50.4% compared to control. Significantly less reduction (35 to 40%) was exhibited by LE 118, LE 57, LE 114, and LE 27 showing their somewhat tolerant nature toward drought stress (Fig. 2). Therefore, it can be clearly stated that water deficit, as a result of soil drying, caused a major adverse effect on yield and yield components even in tolerant genotypes. The present study confirms previous findings by Farooq *et al*. (2009) and Manjunatha *et al*. (2004). Doorenbos and Kassam (1979) indicated that the highest demand for water supply in tomato plants occurs at the flowering phase. Water deficit during this stage would have reduced the number of flowers produced and, as suggested by Mahendran and Bandara (2000), limitation of water at flowering stage not only reduces flower formation but also increases flower shedding.

Purseglove *et al*. (1981) stated that, although the cultivar has a dominant influence over quality determinant properties, the environment in which it grows also has a significant role in the quality characters. Fruit quality, mainly total soluble solids, vitamin C, and acid contents have been reported to change under moisture stress (Kozlowski, 1972). However, in the present study, a slight enhancement in ascorbic acid content was noticed in all the genotypes in response to drought stress. Furthermore, TSS, Lycopene and citric acid content of the fruit also increased slightly. Our work corroborates earlier findings by Ali *et al*. (1980) in tomato. Also Nahar *et al*. (2011) explained that the fruit quality improvement under water deficit conditions in tomato might be due to the synthesis of ascorbic, citric, and malic acid. In the present study, LE 118, LE 57, and LE 27 showed their primacy with the highest ascorbic acid content, as well as higher TSS and lycopene content. This finding is strongly supported by Tambussi *et al*. (2000), who also reported that the increase in ascorbic acid might be an effective strategy to protect membranes from oxidative damage in water stressed condition.

From perusal of the results obtained for SPAD value, soluble protein, SPS activity, fruit characters, lycopene, ascorbic acid, TSS and yield, it can be inferred that genotypes LE 114, LE 57, LE 118, and LE 27 performed better under drought conditions and can be categorized as drought tolerant genotypes compared to genotypes LE 1 and LE 125, drought susceptible ones. However, further studies are required to confirm the results by molecular evidence. The tolerant genotypes could be utilized for further breeding programmes to evolve new tomato genotypes for better drought tolerance with higher yield.

Acknowledgements

The authors wish to express their sincere thanks to the Professor and Head, Department of Vegetable Science, for providing seed materials for this research and all the faculty members of the Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore for providing valuable input for the research work.

References

ALI A.K., DELBERT W.H., WILLIAM O.P., 1980 - *Evaluating leaf water potential, stomatal resistance and canopy surface temperature of tomatoes as indices for irrigation timing*. - Acta Horticulturae, 100: 181-192.

ALONI B., KARNI L., ZAIDMAN Z., SCHAFFER A.A., 1996 - *Changes of carbohydrates in pepper (*Capsicum annuum *L.) owers in relation to their abscission under different shading regimes*. - Ann. Bot., 78: 163-168.

ASHWELL G., 1957 - *Colorimetric analysis of sugars,* pp. 73-105. - In: COLOWICK S.P., and N.O. KAPLAN *(*eds.*) Methods in Enzymology*, III. Academic Press, New York, USA.

BARTHOLOMEW D.M., BARTLEY G.E., SCOLNIK P.A., 1991 - *Abscisic-acid control of rbcS and cab transcript ion in tomato leaves.* - Plant Physiol., 96: 291-296.

BHATT R.M., RAO N.K.S., UPRETI K.K., SHOBHA H.S., 2009 - *Floral abscission and changes in sucrose phosphate synthase and invertase activities in water deficit tomato*. - Indian J. Plant Physiol., 14(4): 370-376.

BOT A.J., NACHTERGAELE F.O., YOUNG A., 2000 - *Land resource potential and constraints at regional and country levels*. - World Soil Resources Reports 90, Land and Water Development Division, FAO, Rome.

BOUTRAA T., 2010 - *Improvement of water use efficiency in irrigated agriculture: A review.* - J. Agron., 9: 1-8.

DANIEL C., TRIBOI E., 2002 - *Changes in wheat protein aggregation during grain development: Effects of temperature and water stress*. - Eur. J. Agron., 16: 1-12.

DIETHELM R., SHIBLES R., 1989 - *Relationship of enhanced sink demand with photosynthesis and amount and activity of ribulose 1,5-bisphosphate carboxylase in soybean leaves.* - J. Plant Physiol., 134: 70-74.

DOORENBOS J., KASSAM A.H., 1979 - *Yield response to water.* - Food and Agriculture Organization of the United Nations, Rome.

ETSUSHI K., TAKUYA A., FUMITAKE K., 2009 - *Correlation of Chlorophyll meter readings with gas exchange and chlorophyll fluorescence in flag leaves of rice (*Oryza sativa *L.) plants*. - Plant Prod. Sci., 12(1): 50-53.

FAROOQ M., WAHID A., KOBAYASHI N., FUJITA D., BASRA S.M.A., 2009 - *Plant drought stress: effects, mechanisms and management.* - Agron. Sustain. Dev., 29: 185-212.

FOYER C.H., VALADIER M.H., MIGGE A., BECKER T.W., 1998 - *Drought induced effects on nitrate reductase activity and mRNA on the coordination of nitrogen and carbon metabolism in maize leaves.* - Plant Physiol., 117: 283-292.

GOMEZ K.A., GOMEZ A.A., 1984 - *Statistical procedures for agricultural research.* - 2nd Ed. John Wiley and Sons, New York, USA, pp. 680.

HAWKINS T.S., GARDINER E.S., COMER G.S., 2009 - *Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research*. - J. Nature Conservation, 17: 123-127.

HUBER S.C., HUBER J.L., 1996 - *Role and regulation of sucrose-phosphate synthase in higher plant*. - Ann. Rev. Plant Physiol. Plant Mol. Biol., 47: 431-444.

ISOPP H., FREHNER M., LONG S.P., NOSBERGER J., 2000 - *Sucrose phosphate synthase responds differently to source-sink relations and photosynthetic rates:* Lollium perenne *L. growing at elevated CO_2 in the field.* - Plant Cell Environ., 23: 597-607.

JOSEPH M.C., RANDALL D.D., NELSON C.J., 1981 - *Photosynthesis and RUBP-case of polyploidy tall fescue.* - Plant Physiol., 68: 894-898.

KOKUBUN M., SHIMADA S., TAKAHASHI M., 2001 - *Flower abortion caused by preanthesis water deficit is not attributed to impairment of pollen in soybean.* - Crop Sci., 4: 1517-1521.

KOZLOWSKI T.T., 1972 - *Water deficit and plant growth.* - Academic Press, London, UK, pp. 91-111.

LOWRY O.H., BROUGHT N.T.R., FARR L.A., RANDALL R.J., 1951 - *Protein measurement with folin phenol reagent.* - J. Biol. Chem., 193: 265-275.

MA B.L., MORRISON M.J., VOLDENG H.D., 1995 - *Leaf greenness and photosynthetic rates in soybean.* - Crop Sci., 35: 1411-1414.

MAHENDRAN S., BANDARA D.C., 2000 - *Effects of soil moisture stress at different growth stages on vitamin C, capsaicin, and ß-carotene contents of chilli (*Capsicum annum *L.) fruits and their impact on yield.* - Trop. Agric. Res., 12: 95-106.

MANJUNATHA M.V., RAJKUMAR G.R., HEBBARA M., RAVISHANKAR G., 2004 - *Effect of drip and surface irrigation on yield and water-production efficiency of brinjal (*Solanum melongena*) in saline vertisols.* - Indian J. Agric. Sci., 74 (11): 583-587.

NAHAR K., ULLAH SM., ISLAM N., 2011 - *Osmotic adjustment and quality response of five tomato cultivars (*Lycopersicon esculentum *Mill.) following water deficit stress under subtropical climate.* - Asian J. Plant Sci., 10: 153-157

NIEDZWIEDZ-SIEGEN I., BOGATEK-LESZCZYNSKA R., COME D., CORBINEAU F., 2004 - *Effects of drying rate on dehydration sensitivity of excised wheat seedling shoots as related to sucrose metabolism and antioxidant enzyme activities.* - Plant Sci., 167: 879-888.

NOGGLE G.R., FRITZ G.J., 1986 - *Introductory plant physiology.* - Prentice Hall of India Pvt. Ltd, New Delhi, India, pp. 171.

PAVLINOVA O.A., BALAKHONTSEV E.N., PRASOLOVA M.F., TURKINA M.V., 2002 - *Sucrose phosphate synthase, sucrose synthase and invertase in sugar beet leaves.* - Russ. J. Plant Physiol., 49: 68-73.

PENG S., GARCIA F.V., LAZA R.C., SANICO A.L., VISPERAS R.M., CASSMAN K.G., 1996 - *Increased N-use efficiency using a chlorophyll meter on high-yielding irrigated rice.* - Field Crops Res., 47: 243-252.

PURSEGLOVE J.W., BROWN E.G., GREEN C.L., ROBBINS

S.R.J., 1981 - *Spices*. *Tropical Agriculture Series. Vol. 1* - Longman Group Ltd., London, UK, pp. 450.

RANGANNA S., 1986 - *Handbook of analysis and quality control for fruit and vegetable products*. - 2nd ed. Tata Mc Graw Hill Publication Co. Ltd, New Delhi, India.

RUIZ R., GUARDIOLA J.L., 1994 - *Carbohydrate and mineral nutrition of orange fruitlets in relation to growth and abscission*. - Physiol. Plant., 90: 27-33.

SADASIVAM S., MANICKAM A., 1996 - *Biochemical methods*. - 2nd Edition New Age International publishers, pp. 59-66.

SRIVASTAVA K., KUMAR S., PRAKASH P., VAISHAMPAYAN A., 2012 - *Screening of tomato genotypes for reproductive characters under high temperature stress conditions.* - Sabrao J. Breed. Genet., 44(2): 263-276.

TAMBUSSI E.A., BARTOLI C.G., BELTRANO J., GUIAMET J.J., ARAUS J.L., 2000 - *Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*).* - Physiol. Plant., 108: 398-404.

VASSEY T.L., SHARKEY T.D., 1989 - *Mild water stress of* Phaseolus vulgaris *plants leads to reduced starch synthesis and extractable sucrose phosphate synthase activity.* - Plant Physiol., 89: 1066-1070.

VU J.C.V., GESCH R.W., ALLEN L.H., BOOTE K.J., BOWES G., 1999 - *CO_2 enrichment delays a rapid, drought-induced decrease in Rubisco small subunit transcript abundance*. - J. Plant Physiol., 155: 139-142.

WARDLAW I.F., WILLENBRINK J., 1994 - *Carbohydrate storage and mobilization by the culm of wheat between heading and grain maturity: the relation to sucrose synthase and sucrose phosphate synthase.* - Aus. J. Plant Physiol., 21: 255-271.

WIEN H.C., TURNER A.D., YANG S.F., 1989 - *Hormonal basis for low light intensity-induced flower bud abscission of pepper*. - J. Amer. Soc. Hort. Sci., 114: 981-985.

WILLIAMS S.J., BULMAN M.P., NEILL S.J., 1994 - *Wilt-induced ABA biosynthesis, gene-expression and down-regulation of rbcS messenger RNA levels in* Arabidopsis thaliana. - Physiol. Plant., 91: 177-182.

XU Z.Z., ZHOU G.S., 2006 - *Combined effects of water stress and high temperature on photosynthesis, nitrogen metabolism and lipid peroxidation of perennial grass* Leymus chinensis*.* - Planta, 224: 1080-1090.

YANG J., ZHANG J., WANG Z., ZHU Q., LIJUN L., 2002 - *Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilisation of carbon reserves in rice subjected to water stress during grain filling*. - Planta, 215: 645-652.

ZRENNER R., STITT M., 1991 - *Comparison of the effect of rapidly and gradually developing water-stress on carbohydrate metabolism in spinach leaves.* - Plant Cell Environ., 14(9): 939-946.

Adv. Hort. Sci., 2016 30(1): 13-21

DNA changes in cotton (*Gossypium hirsutum* L.) under salt stress as revealed by RAPD marker

B. Saleh
Department of Molecular Biology and Biotechnology, AECS, P.O. Box 6091, Damascus, Syria.

Key words: cotton, genomic template stability, RAPD, salt stress.

Abstract: Random amplified polymorphic DNA (RAPD) analysis was applied to evaluate DNA changes among four upland cotton (*Gossypium hirsutum* L.) varieties [Niab 78 (N78), Deir-Ezzor 22 (DE22), Deltapine 50 (DP50) and Aleppo 118 (A118)] grown under non-saline conditions (control) and salt stress (200 mM NaCl) for seven weeks. Changes in RAPD profiles were measured as genomic template stability (GTS%). The highest estimated GTS% value was recorded for the two sensitive varieties, DP50 (79.1%) followed by A118 (58.2%); whereas, the lowest value was recorded for the two other tolerant varieties DE22 (36.7%) followed by N78 (26.4%). Based upon the data presented, RAPD marker could be used as potential tool for early identification of cotton tolerance to salt stress.

1. Introduction

Cotton is an economically important plant grown world-wide as a principal source of staple fiber and vegetable oil. A great deal of effort has been made to improve cotton cultivation and characteristics by breeders. Cotton is one of the major fiber crops in Syria, with a cultivated area of 125,000 ha, a production of 470,000 t of seed cotton, and lint production at 160,000 t. Yarn spinning capacity is estimated at 180,000 t (USDA, 2011). Salinity tolerance is a complex trait that involves physiological, biochemical, cellular, and genetic strategies. At present, out of 1.5 billion ha of cultivated land around the world, about 77 million ha (5%) is affected by excess salt content (Moradi *et al*., 2011). There is evidence that high salt concentrations cause an imbalance of the cellular ions resulting in ion toxicity and osmotic stress, leading to the generation of reactive oxygen species (ROS) which alter cellular metabolism causing lipid peroxidation, protein denaturing, and DNA mutation (Dat *et al*., 2000; Davenport *et al*., 2003; Implay, 2003). Moreover, salt stress causes nuclear deformation and subsequent nuclear degradation (Katsuhara and Kawaski, 1996). Structural changes of nuclei caused by salt stress have been previously reported as well (Werker *et al*., 1983).

At present, there are several methods (physiological, biochemical, and molecular) available for detecting different kinds of DNA damage but with some limitations. Recently, molecular markers have been successfully applied to detect DNA damage induced by different abiotic stresses, particularly salinity. Among others, RAPD technique has been well documented as a sensitive means of detecting DNA damage and shows potential as a reliable and reproducible assay for the detection of DNA fragmentation and chromosomal mutations (Citterio *et al*., 2002).

The RAPD marker has been extensively applied for salinity tolerance screening in plant breeding programs, such as in date palm (*Phoenix dactylifera* L.) (Kurup *et al*., 2009), aquatic plants *Hydrilla verticillata* and *Ceratophyllum demersum* (Gupta and Sarin, 2009), in *Euplotes vannus* (Protozoa, Ciliophora) (Zhou *et al*., 2011), and in *Acacia Senegal* (Daffalla *et al*., 2011); in cotton (Dojan *et al*., 2012) and in fish full-sib Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*) and their diallel interspecific hybridization (El-Zaeem, 2012); and recently, also in soybean (*Glycine max* L.) (Khan *et al*., 2013).

RAPD bands can be scored for genomic template stability (GTS) evaluation to detect various types of DNA damage and mutations (rearrangement, point mutations, small insertions or deletions of DNA and polyploidy changes) which suggests that RAPD bands may potentially form the basis of novel biomarker

(*) Corresponding author: ascientific@aec.org.sy
Received for publication 28 May 2015
Accepted for publication 20 December 2015

assays for the detection of DNA damage and mutations in the cells of bacteria, plants, and animals (Savva, 1998; Atienzar *et al*., 1999; Tanee *et al*., 2012). It is well documented that genomic template stability ratios (GTS) were calculated. GTS implies qualitative measure reflecting changes in RAPD profiles. Changes in RAPD and profiles were expressed as reductions in GTS in relation to profiles obtained from control samples (Gupta and Sarin, 2009; Aly, 2012; Dojan *et al*., 2012; Tanee *et al*., 2012).

Therefore, this investigation aimed to detect DNA changes induced by NaCl application by monitoring the RAPD profiles of control and stressed plants in four upland cotton (*Gossypium hirsutum* L.) varieties grown in Syria.

2. Materials and Methods

Plant materials and growth conditions

Two local varieties were selected on the basis of their wide-ranging tolerance towards salinity: Deir-Ezzor 22 (DE22) as salt-tolerant and Aleppo 118 (A118) as salt-sensitive variety (Saleh, 2011). These two varieties were compared with two introduced cotton varieties, Niab 78 (N78) (known as salt-tolerant) and Deltapine 50 (DP50) (known as salt-sensitive) under 0 and 200 mM NaCl for seven weeks. Seeds of upland cotton (*G. hirsutum* L.) were provided by the General Commission for Scientific Agricultural Research of Syria (GCSAR).

Seeds were soaked in distilled water for 24 h and then planted in pots filled with a 1:2 (v/v) mixture of perlite:peatmoss. Germination was carried out in a greenhouse at 18°C, 12 h photoperiod, and relative humidity of 80%. Seedlings were allowed to grow in a greenhouse under controlled conditions (temperature 25°C, 12 h photoperiod, and relative humidity 80%). Seedlings were irrigated with tap water for one week before the initiation of NaCl treatments. Salt stress application was carried out by adding NaCl (200 mM) to the water. Plants were irrigated twice a week with water with or without salt. All solutions were changed twice a week. The same environmental conditions were maintained during the experiment. The experiment (five replicates/treatment) was carried out in the greenhouse for seven weeks.

Genomic DNA extraction

Plant genomic DNA was extracted from young leaves (bulk of five plants/variety) including the control and stressed plants (200 mM NaCl) using CTAB (cetyltrimethylammonium bromide) protocols described by Doyle and Doyle (1987) with minor modifications.

Leaf tissue (150 mg) was ground in liquid nitrogen and the powder was transferred to a 2 ml Eppendorf tube, mixed with 900 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.0018 ml β-mercaptoethanol, 2% CTAB), and incubated at 65°C for 20 min. One volume of a chloroform:isoamyl alcohol mix (24:1, v/v) was added and centrifuged at 12,000 *g* for 10 min at 4°C. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with an equal volume of cold isopropanol and kept at -20°C for 10 min. It was then centrifuged at 12,000 *g* for 10 min at 4°C, the supernatant was discarded, and DNA was spooled out and washed with 1 M ammonium acetate and 100% ethanol. The cleaned DNA pellet was air dried and dissolved in 100 µl of 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Finally 5 µl of RNase (10 mg ml^{-1}) were added and incubation for 30 min at 37°C was applied. DNA concentration was quantified by DNA Spectrophotometer at 260/280 nm and adjusted to final concentration of 10 ng μl^{-1}. DNA was stored at -80°C until needed.

RAPD marker

Twenty-three RAPD primers from Operon Technologies Inc. (USA) and three primers from the University of British Columbia were tested to detect DNA changes in stressed plants, and their respective controls, for four cotton varieties.

RAPD marker was performed as described by Williams *et al*. (1990) with a minor modification. PCR amplification reaction was carried out in 25 µl reaction volume containing 1xPCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 25 pmol primer, 1.5 U of Taq DNA polymerase and 30 ng template DNA. PCR amplification was performed in a T-gradient thermal cycler (Bio-Rad; T Gradient) programmed to fulfill 42 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step (1 min at 94°C), an annealing step (2 min at 35°C), and an extension step (for 2 min at 72°C). A final extension cycle was performed for 7 min at 72°C. The PCR products were separated on a 1.5% ethidium bromide-stained agarose gel (Bio-Rad) in 0.5xTBE buffer. Electrophoresis was performed for 3 h at 85V and visualized with a UV transilluminator. Band sizes were determined by comparison with a 1 kb DNA Ladder Mix, ready for use (Fermentas).

RAPD data analysis and Genomic Template Stability (GTS) estimations

DNA changes induced in treated plants compared to their respective controls were screened by RAPD assay. The polymorphism was calculated in relation to the appearance of new bands and disappearance of bands in treated plants, compared to control band patterns.

Genomic template stability (GTS%) was calculated as follows:

$$GST\% = (1- a/n) \times 100$$

where (a) is the average number of changes in the DNA profile and (n) the number of total bands in the control. Polymorphism observed in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison to the cotrol RAPD profiles (Atienzar *et al*., 2002).

3. Results

A set of 26 random10-mer primers was used to detect the DNA changes among four cotton varieties under salt stress application compared to their respective controls. RAPD fragment sizes ranged from 200 to 3000 bp. The generated band characteristics for the four varieties (including control and stressed plants) are summarized in Table 1. The total number of characteristic bands (common observed bands in control and stressed plants for the four examined varieties) was 29. The amplification products produced from 26 RAPD primers are listed in Table 2 in terms of loss or appearance of new bands (number and size) under salt stress compared to their respective controls for each variety separately. The RAPD analysis carried out on the four cotton varieties produced a number of distinct fragments which varied according to each tested primer. Twelve of the 26 RAPD primers (OPA02, OPB05, OPC08, OPD08, OPD20, OPJ07, OPK13, OPK17, OPR12, OPY10, UBC132 and UBC159) produced polymorphic bands under saline conditions for the four tested varieties (Table 2).

Figure 1 shows the amplification products using OPA02, OPB17 and OPY10 RAPD primers with template DNA from the four varieties under control and saline conditions (200 mM NaCl).

Changes in DNA pattern induced by NaCl treatment in the four tested cotton varieties were detected based on estimated genomic template stability (GTS%) (Table 3). In this respect, it was found that the highest GTS% was recorded in salt-sensitive cotton, whereas the lowest was found among salt-tolerant varieties (Table 3).

Table 1 - Characteristic bands identified for the four tested cotton varieties using 26 RAPD primers

Primer name	Sequence (5' - 3')	Characteristic bands (number and size)
OPA02	TGCCGAGCTG	(1) 950
OPA04	AATCGGGCTG	(3) 250, 550 & 1600
OPB05	TGCGCCCTTC	(1) 1500
OPB17	AGGGAACGAG	0
OPC08	TGGACCGGTG	(2) 500 & 800
OPC13	AAGCCTCGTC	(1) 450
OPD08	GTGTGCCCCA	(1) 1200
OPD20	GGTCTACACC	0
OPE07	AGATGCAGCC	(3) 550, 650 & 800
OPE15	ACGCACAACC	0
OPG11	TGCCCGTCGT	(3) 700, 1200 & 2100
OPJ01	CCCGGCATAA	0
OPJ07	CCTCTCGACA	0
OPK12	TGGCCCTCAC	0
OPK13	GGTTGTACCC	0
OPK17	CCCAGCTGTG	0
OPQ01	GGGACGATGG	(3) 450, 600 & 1000
OPQ18	AGGCTGGGTG	(3) 650, 750 & 1200
OPR09	TGAGCACGAG	(1) 250
OPR12	ACAGGTGCGT	(1) 300
OPT18	GATGCCAGAC	(2) 1200 & 1350
OPW17	GTCCTGGGTT	(1) 2000
OPY10	CAAACGTGGG	(1) 650
UBC132	AGGGATCTCC	0
UBC159	GAGCCCGTAG	(1) 1600
UBC702	GGGAGAAGGG	(1) 400
Total		29

4. Discussion and Conclusions

Detection of DNA changes in cotton via salt stress was assessed using RAPD marker system. As shown in Table 1, characteristic bands ranged between 0 (OPB17, OPD20, OPE15, OPJ01, OPJ07, OPK12, OPK13, OPK17 and UBC132) and 2 (OPC08 and OPT18), whereas the highest number (three) was yielded by OPA04, OPE07, OPG11, OPQ01 and OPQ18 RAPD primers (Table 1). Our findings reveal that nine out of the 26 tested RAPD primers generat-

Table 2 - Markers identified by 26 RAPD primers for the four tested cotton varieties under salt stress compared to their respective controls. DNA changes induced by NaCl treatment using RAPD marker as described by loss or appearance of new bands (number and size) under salt stress compared to their respective controls for each variety separately

Primer name	N78		DE22		DP50		A118		Total polymorphic bands
	C	T	C	T	C	T	C	T	
OPA02	9		8		9		9		
-		(4) 400, 650, 1800 & 2500		(3) 650, 800 & 2500		(2) 1800 & 2500		(2) 1800 & 2500	16
+		(2) 600 & 1500		(3) 500, 600 & 900		ND		ND	
OPA04	3		3		3		3		
-		ND		ND		ND		ND	5
+		(3) 400, 500 & 1000		(2) 400 & 500		ND		ND	
OPB05	5		5		4		5		
-		(4) 450, 650, 1000 & 2000		(3) 450, 1000 & 2000		(1) 900		(1) 2000	14
+		(3) 500, 700 & 1100		(2) 500 & 1100		ND		ND	
OPB17	7		9		8		8		
-		(5) 600, 700, 1100, 1200 & 1800		(4) 350, 800, 1500 & 1800		ND		(5) 400, 600, 800, 1000 & 1800	20
+		(2) 400 & 650		(2) 700 & 1100		ND		(2) 450 & 750	
OPC08	4		5		5		4		
-		(2) 1850 & 1900		(1) 1200		(1) 1850		ND	10
+		(2) 300 & 900		(2) 1350 & 1900		ND		(2) 1850 & 1900	
OPC13	6		6		5		5		
-		(1) 1000		(2) 500 & 1000		ND		ND	12
+		(5) 300, 800, 900, 1100 & 1350		(4) 300, 1100, 1350 & 1500		ND		ND	
OPD08	4		3		4		3		
-		(2) 450 & 700		(2) 450 & 750		(1) 900		(2) 450 & 800	15
+		(2) 650 & 1350		(4) 500, 600, 800 & 1350		(1) 800		(1) 600	
OPD20	6		4		2		2		
-		(4) 650, 850, 1100 & 1850		(3) 700, 900 & 1350		(1) 800		(1) 300	13
+		(1) 1350		(1) 800		(1) 300		(1) 200	
OPE07	8		8		8		8		
-		(5) 1000, 1300, 1800, 2300 & 3000		(1) 1800		ND		ND	7
+		(1) 1100		ND		ND		ND	
OPE15	5		4		6		6		
-		(1) 200		(2) 800 & 1800		ND		ND	8
+		(4) 800,1300, 1500 & 1800		(1)1500		ND		ND	
OPG11	3		3		3		3		
-		ND		ND		ND		ND	2
+		(1) 600		(1) 600		ND		ND	
OPJ01	7		6		7		7		
-		(5) 400, 600, 850, 1200 & 2000		(3) 400, 600 & 850		ND		(2) 600 & 2000	20
+		(4) 350, 500, 950 & 1800		(4) 350, 500, 950 & 1800		ND		(2) 500 & 800	
OPJ07	3		4		2		3		
-		(3) 500, 800 & 1600		(4) 300, 500, 700 & 1600		(2) 550 & 1350		(1) 1100	21
+		(3) 700, 900 & 1200		(3) 550, 1000 & 1200		(2) 450 & 1600		(3) 450, 550 & 650	

T(-) loss bands, (+) gains bands, (ND) no differences.

to be continued

Table 2 (continued)

Primer name	N78		DE22		DP50		A118		Total polymorphic bands
	C	T	C	T	C	T	C	T	
OPK12	3		3		2		2		
-		(3) 700, 900 & 1350		(3) 800, 900 & 1350		ND		ND	11
+		(3) 850, 950 & 1100		(2) 500 & 1000		ND		ND	
OPK13	4		4		3		3		
-		(2) 650 & 1100		(2) 350 & 1100		(1) 200		(1) 490	20
+		(4) 450, 750, 1200 & 1350		(4) 500, 750, 1200 & 1350		(3) 300, 500 & 1100		(3) 700, 1200 & 1350	
OPK17	8		4		4		6		
-		(4) 400, 1500, 1850 & 2500		(1) 1850		(2) 700 & 1000		(4) 300, 400, 1000 & 1850	19
+		(3) 300, 350 & 800		(2) 350 & 1200		(2) 500 & 1500		(1) 1500	
OPQ01	9		4		4		4		
-		(5) 300, 800, 1100, 1200 & 1800		(1) 1100		ND		ND	10
+		(3) 200, 700 & 900		(1) 300		ND		ND	
OPQ18	7		3		6		6		
-		(4) 650, 900, 1350 & 1500		ND		ND		ND	9
+		(2) 550 & 1600		(2) 1500 & 1600		ND		(1) 1600	
OPR09	6		3		4		4		
-		(4) 400, 550, 1100 & 3000		(1) 600		ND		(1) 600	9
+		(2) 600 & 900		(1) 1000		ND		ND	
OPR12	11		10		7		7		
-		(6) 200, 450, 600, 1100, 2100 & 2250		(6) 250, 500, 800, 900, 1350 & 2250		(1) 900		(3) 200, 1200 & 1350	28
+		(4) 250, 500, 900 & 1800		(4) 700, 1000, 1100 & 1800		(2) 1350 & 2250		(2) 450 & 1000	
OPT18	5		8		10		10		
-		(1) 650		(2) 1800 & 2000		ND		ND	9
+		(4) 400, 500, 1000 & 2250		(1) 950		ND		(1) 2000	
OPW17	5		4		3		3		
-		(2) 600 & 1200		(3) 300, 900 & 1500		ND		ND	17
+		(5) 450, 500, 800, 1350 & 1500		(4) 300, 450, 500 & 800		ND		(3) 250, 450 & 800	
OPY10	4		5		4		4		
-		(1) 3000		(2) 1500 & 3000		(1) 400		(3) 400, 900 & 2500	12
+		(3) 400, 850 & 2500		(2) 400 & 2500		ND		ND	
UBC132	5		5		4		5		
-		(4) 1000, 1350, 1500 & 3000		(3) 550, 600 & 3000		(1) 1200		(3) 400, 1000 & 1200	17
+		(3) 600, 1200 & 1800		(3) 1000, 1350 & 1500		ND		ND	
UBC159	3		6		3		3		
-		(1) 500		(2) 1350 & 2000		(2) 500 & 700		(2) 650 & 2000	19
+		(5) 550, 700, 950, 1100 & 1350		(3) 300, 700 & 900		(2) 450 & 600		(2) 500 & 700	
UBC702	3		3		3		3		
-		(1) 800		(2) 750 & 800		ND		ND	7
+		(2) 600 & 2500		(1) 2500		ND		(1) 2500	

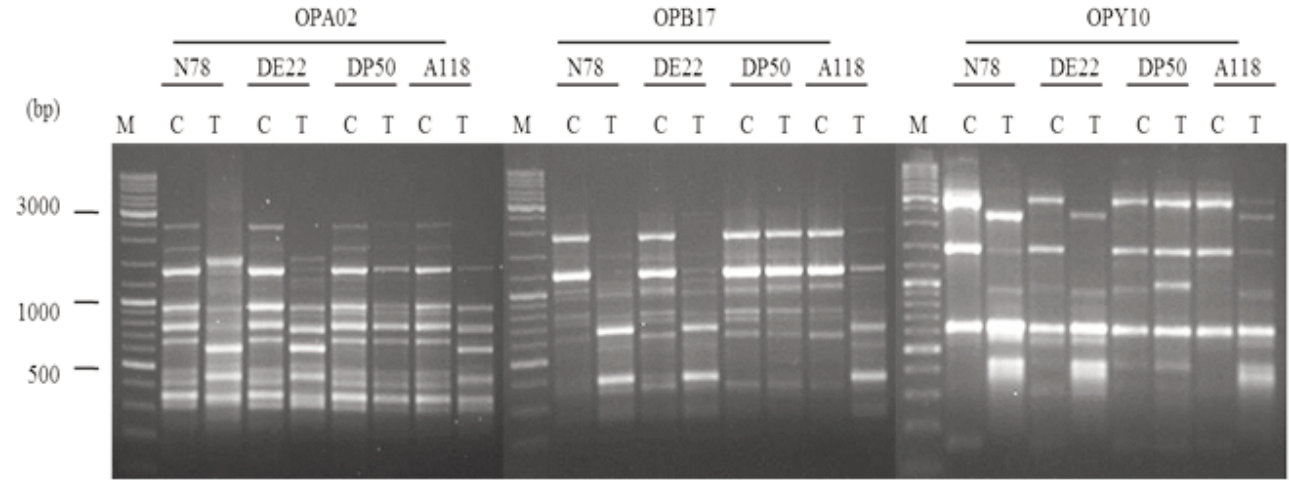

Fig. 1 - RAPD banding profiles generated by OPA02, OPB17 and OPY10 primers in the four tested cotton varieties showing DNA changes induced by NaCl application for seven weeks, C: Control, T: Treated plants. M: 1 kb DNA Ladder Mix, ready for use.

Table 3 - Genomic Template Stability (GTS%) estimated by 26 RAPD primers for the four tested cotton varieties under salt stress compared to their respective controls

Primer name	Control	N78 200 mM NaCl	DE22 200 mM NaCl	DP50 200 mM NaCl	A118 200 mM NaCl
OPA02	100	33.3	25	77.8	77.8
OPA04	100	0	33.3	100	100
OPB05	100	40	0	75	80
OPB17	100	0	33.3	100	12.5
OPC08	100	0	40	80	50
OPC13	100	0	0	100	100
OPD08	100	0	100	50	0
OPD20	100	16.7	0	0	0
OPE07	100	25	87.5	100	100
OPE15	100	0	25	100	100
OPG11	100	66.7	66.7	100	100
OPJ01	100	28.6	16.7	100	42.9
OPJ07	100	100	75	100	33.3
OPK12	100	100	66.7	100	100
OPK13	100	50	50	33.3	33.3
OPK17	100	12.5	25	0	16.7
OPQ01	100	11.1	50	100	100
OPQ18	100	14.3	33.3	100	83.3
OPR09	100	0	33.3	100	75
OPR12	100	9.1	0	57.2	28.6
OPT18	100	0	62.5	100	90
OPW17	100	40	75	100	0
OPY10	100	0	20	75	50
UBC132	100	40	20	75	40
UBC159	100	100	16.7	33.3	33.3
UBC702	100	0	0	100	66.7
Mean	100	26.4	36.7	79.1	58.2

ed no characteristic bands for the for tested cotton varieties (Table 1). It is worth noting that primer OPR12 identified more polymorphisms (28) than any other primer tested (ranging between two for primer OPG11 and 21 for primer OPJ07) (Table 2). Whereas, the banding patterns produced by primers OPA04, OPC13, OPE07, OPE15, OPG11, OPK12 and OPQ01 were not polymorphic for varieties DP50 and A118 (Table 2).

Another investigation demonstrated varietal variation in salt tolerance among these cotton varieties based on various examined physiological indices (Saleh, 2011). According to the study, the DE22 variety could relatively be classified as salt tolerant variety to other tested varieties. Dojan *et al*. (2012) reported the potential of RAPD markers for the detection of DNA damage induced by NaCl in cotton.

Likewise, the RAPD marker has the potential to be applied in environmental pollution detection, e.g. Gupta and Sarin (2009) applied the same marker to detect pollution by cadmium (Cd) in two aquatic plants. Zhou *et al*. (2011) also used RAPD bands to indicate DNA damage in *Euplotes vannus* (Protozoa, Ciliophora) induced by nitrofurazone in marine ciliates. Previously, Aly (2012) used the same marker for genotoxic effect detection of Cd stress on Egyptian clover and Sudan grass plants.

Changes in RAPD profiles were also measured as Genomic Template Stability (GTS) and the data suggest noticeable genomic template instability (Table 3). Reduction in GTS values was observed under salt stress, compared to their respective controls for the four tested varieties (Table 3). Similarly, genetic instability induced by NaCl treatment of cotton was reflected by changes in RAPD profiles: disappearance

of bands and appearance of new bands occurred in the profiles in comparison to those of the controls (Fig. 1, Table 3). Our data supports the suggestion by Dojan *et al*. (2012) that detected DNA changes, induced by NaCl, using RAPD marker could be explained as previously reported by Atienzar *et al*. (1999).

It has been demonstrated that DNA damage levels could be reflected in GTS (Atienzar *et al*., 1999). The later investigation suggested that the loss of bands may be attributed to genomic rearrangements or to point mutations causing alterations in oligonucleotide priming sites, while appearance of new bands could be related to the presence of oligonucleotide priming sites which become accessible to oligonucleotide primers after structural changes (DNA mutation, deletions or homologous recombination).

Table 3 reveals that GTS% values decreased with salt application for the four tested varieties. Our data show that the highest estimated GTS value was recorded for the two sensitive varieties, DP5 (79.1%) followed by A118 (58.2%); whereas, the lowest was recorded for the two tolerant varieties, N78 (26.4%) followed by DE22 (36.7%) (Table 3).

Gupta and Sarin (2009) reported that the genomic template stability test was significantly affected by heavy metal stress, while Aly (2012) reported that GTS values decreased obviously with an increase in cadmium (Cd) concentration in Egyptian clover and Sudan grass seedlings. On the other hand, Tanee *et al*. (2012) used GTS to identify the *Vanda* species (Orchidaceae) of Thailand. Our results are in accordance with Dojan *et al*. (2012) who reported that there is positive correlation between GTS and other parameters (stem and leaf growth and stem length) under NaCl stress in cotton.

In this respect, the estimated GTS values in the current investigation were positively correlated with various physiological indices (biomass and leaf K^+/Na^+ ratio) tested under NaCl application in cotton (Saleh, 2011). Moreover, a positive relationship was also determined between GTS values and recent findings (Saleh, 2013) based on physiological indices (relative water content, osmotic potential and salt tolerance index) among the same tested varieties (Dojan *et al*., 2012).

Overall, the lowest estimated GTS values combined with the highest polymorphism level recorded for the tolerant varieties (N78 and DE22) (where, % polymorphic level was 68.5, 60.9, 21.3 and 36.4% for N78, DE22, DP50 and A118, respectively exposed to 200 mM NaCl for 7 weeks, using the same marker) could explain their salinity tolerance compared to the other tested varieties. However, the lowest estimated GTS value recorded for N78 and DE22 varieties could be attributed to genetic variation, inducing new protein in relation to salinity tolerance.

It has been successfully demonstrated that environmental constraint induced variation in DNA methylation pattern as a developed epigenetic mechanism after exposure to abiotic stress (Zhong and Wang, 2007; Peng and Zhang, 2009). Our findings could be supported by the data provided in Zhong and Wang (2007), where genotyping variation in wheat (*Triticum aestivum* L.) cultivar salinity tolerance was reported. In this respect, the study mentioned that the salt-sensitive wheat variety had a lower methylation rate compared to salt-tolerant ones.

Recently, Saleh (2013) reported that N78 and DE22 varieties showed a better protection mechanism against salinity damage than the other tested varieties, demonstrating variation in salt tolerance among cotton varieties based on physiological indices. Likewise, in the same investigation comparing the protein profiles between control plants and those salts treated using SDS-PAGE showed protein changes under salt treatment compared to their respective control. In this respect, the expression of ~19, ~21 and ~26 kDa for N78 and ~21 kDa protein for DE22, was highly increased by salt treatment, indicating that it could play a role in salt stress response. On the other hand, newly synthesized protein of ~30 kDa was recorded for both DE22 and N78 varieties under saline treatment which was not observed in their respective controls. The other two tested varieties (DP50 and A118) showed decreases in the same protein bands (~19, 26 and 34 kDa) under saline conditions, with respect to their respective controls, reflecting their sensitivity to salt stress. Salinity promotes the synthesis of salt stress-specific proteins; many of these proteins were suggested to protect the cell against the adverse effect of salt stress. Accumulation of these proteins is a common response to salt stress (Kong-Ngern *et al*., 2005; Metwali *et al.*, 2011).

It is worth noting that identified bands in DE22 and N78 (salt-tolerant varieties), which were not amplified in salt-sensitive varieties (DP50 and A118), could be related to gene(s) involved in salinity tolerance. These findings are in accordance with Kurup *et al*. (2009).

DNA variation could be exploited in plant breeding programs to improve salinity tolerance in germplasm. Overall, the lowest estimated GTS values for NB78 and DE22 varieties (known as salt-tolerant) reflect their highest polymorphic values. Based on the current results, the RAPD marker was useful to establish specific DNA markers associated with NaCl stress. Therefore, the RAPD marker could be used as useful tool in plant breeding programs for early identification of cotton tolerance to salt stress.

Acknowledgements

The author would like to thank Prof. Othman I, Director General of AECS and Prof. MirAli N, Head of Molecular Biology and Biotechnology Department for their support, and also to the Plant Biotechnology group for technical assistance.

References

ALY A.A., 2012 - *Application of DNA (RAPD) and ultrastructure to detect the effect of cadmium stress in Egyptian clover and Sudan grass plantlets*. - J. Stress Physiol. & Biochem*.,* 8: 241-257.

ATIENZAR F.A., CONRADI M., EVENDEN A.J., JHA A.N., DEPLEDGE M.H., 1999 - *Qualitative assessment of genotoxicity using random amplified polymorphic DNA: Comparison of genomic template stability with key fitness parameters in* Daphnia magna*expose to benzo[a]pyrene*. - Environ. Toxicol. Chem., 18: 2275-2282.

ATIENZAR F.A., VENIR P., JHA A.N., DEPLEDGE M.H., 2002 - *Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations*. - Mut. Res., 521: 151-163.

CITTERIO S., AINA R., LABRA M., GHIANI A., FUMAGALLI P., SGORBATI S., SANTAGOSTINO A., 2002 - *Soil genotoxicity: A new strategy based on biomolecular tools and plants bioindicators*. - Environ. Sci. Technol., 36: 2748-2753.

DAFFALLA H.M., HABEBALLA R.S., ELHADI E.A., KHALFALLA M.M., 2011 - *Random amplified polymorphic DNA (RAPD) marker associated with salt tolerance during seeds germination and growth of selected* Acacia Senegal *provenances*. - African J. Biotech., 10: 5820-5830.

DAT J., VANDENABEELE S., VRANOVA E., VANMONTAGU M., INZE D.,VANBREUSEGEM F., 2000 - *Dual action of the active oxygen species during plant stress responses*. - Cell. Mol. Life Sci., 57: 779-795.

DAVENPORT S.B., GALLEGO S.M., BENAVIDES M.P.,TOMARO M.L., 2003 - *Behavior of antioxidant defense system in the adaptive response to salt stress in* Helianthus annuus *L. cells*. - Plant Growth Regul., 40: 81-88.

DOJAN I., KEKEC G., OZYIGIT I.I., SACKALI M.M., 2012 - *Salinity induced changes in cotton (*Gossypium hirsutum *L.)*. - Pakistan J. Bot., 44: 21-25.

DOYLE J.J., DOYLE J.L., 1987 - *A rapid DNA isolation procedure for small quantities of fresh leaf tissue*.- Phytochemical Bullettin, 19: 11-15.

EL-ZAEEM S.Y., 2012 - *Identification of different selected genotypes of salinity resistance of each of full-sib* Nile tilapia*,* Blue tilapia *and their diallel interspecific hybridization using random amplified polymorphic DNA fingerprinting*. - African J. Biotech., 11: 8645-8652.

GUPTA M., SARIN N.B., 2009 - *Heavy metal induced DNA changes in aquatic macrophytes: Random amplified polymorphic DNA analysis and identification of sequence characterized amplified region marker.* - J. Environ. Sci., 21: 686-690.

IMPLAY J.A., 2003 - *Pathways of oxidative damage*. - The Annu. Rev. Microbiol., 57: 395-418.

KATSUHARA M., KAWASAKI T., 1996 - *Salt stress induced nuclear and DNA degradation in meristematic cells of barley roots*. - Plant Cell Physiol., 37: 169-173.

KHAN F., HAKEEMK.R., SIDDIQIT.O., AHMADA.,2013 - *RAPD markers associated with salt tolerance in soybean genotypes under salt stress*.- Appl. Biochem. Biotech., 170: 257-272.

KONG-NGERN K., DADUANG S., WONGHAM C., BUNNAG S., KOSITTRAKUN M.,THEERAKULPISUT P., 2005 - *Protein profiles in response to salt stress in leaf sheaths of rice seedlings*. - ScienceAsia, 31: 403-408.

KURUP S.S., HEDAR Y.S., AL-DHAHERI M.A., EL-HEAWIETY A.Y., ALY M.A.M., ALHADRAMI G., 2009 - *Morpho-physiological evaluation and RAPD markers-assisted characterization of date palm* (Phoenix dactylifera *L.*) *varieties for salinity tolerance*. - Journal of Food, Agriculture & Environment, 7: 503-507.

METWALI E.M.R., EID M.H., BAYOUMI T.Y., 2011 - *Agronomical traits and biochemical genetic markers associated with salt tolerance in wheat cultivars* (Triticum aestivum *L.*). - Australian J. Basic and Applied Sciences, 5: 174-183.

MORADI A., TAHMOURESPOUR A., HOODAJI M., KHORSANDI F., 2011 - *Effect of salinity on free living - diazotroph and total bacterial populations of two saline soils*. - African J. Microbiol. Res., 5: 144-148.

PENG H., ZHANG J., 2009 - *Plant genomic DNA methylation in response to stresses: Potential applications and challenges in plant breeding*. - Prog. Nat. Sci., 19: 1037-1045.

SALEH B., 2011 - *Effect of salt stress (NaCl) on biomass and K^+/Na^+ ratio in cotton*. - Journal of Stress Physiology & Biochemistry, 7: 5-14.

SALEH B., 2013 - *Water status and protein pattern changes*

towards salt stress in cotton. - Journal of Stress Physiology & Biochemistry, 9: 113-123.

SAVVA D., 1998 - *Use of DNA fingerprinting to detect genotoxic effects*. - Ecotoxicol. Environ. Safe., 41: 103-106.

TANEE T., CHADMUK P., SUDMOON R., ARUNRAT C., NOIKOTR K., 2012 - *Genetic analysis for identification, genomic template stability in hybrids and barcodes of the* Vanda *species (Orchidaceae) of Thailand*. - African J. Biotech., 11: 11772-11781.

USDA, 2011 - *Syria cotton and products annual cotton report.* - GAIN Report, www.fas.usda.gov.

WERKER E., LERNER H.R., WEIMBERG R., POLJAKOOF-MAYBER A., 1983 - *Structural changes occurring in nuclei of barley root cells in response to a combined effect of salinity and ageing*. - Am. J. Bot., 70: 222-225.

WILLIAMS J.G.K., KUBELIK A.R., LIVAK K.J., RAFALSKI A.J., TINGEY S.V., 1990 - *DNA polymorphisms amplified by arbitrary primers are useful as genetic markers*. - Nucleic Acids Res., 18: 6531-6535.

ZHONG L., WANG J.B., 2007 - *The role of DNA hypermethylation in salt resistance of* Triticum aestivum *L*. - Journal of Wuhan Botanical Research, 25: 102-104.

ZHOU L., LI J., LIN X., AL-RASHEID K.A.S., 2011 - *Use of RAPD to detect DNA damage induced by nitrofurazone in marine ciliate* Euplotes vannus (*Protozoa, Ciliophora*). - Aquat. Toxicol., 103: 225-232.

Adv. Hort. Sci., 2016 30(1): 23-29

Vegetable production using a simplified hydroponics system inside City of Dead (Cairo)

A. Giro [1 (*)], S. Ciappellano [2], A. Ferrante [1]

[1] *Dipartimento di Scienze Agrarie e Ambientali Produzione, Territorio, Agroenergia, Università degli Studi di Milano, Via Celoria, 2, 20133 Milano, Italy.*

[2] *Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, Via Celoria, 2, 20133 Milano, Italy.*

Key words: food security, heavy metals, Middle East, sustainable agriculture.

Abstract: This research work was performed in the poorest urban area of Cairo (Egypt) in a slum area called Al-Quarafa. The aim was to develop and evaluate a simplified hydroponics system (HS) to grow vegetables for the local inhabitants who live in an extreme status of food unsafety. In the hydroponic growing system the tomato plants were cultivated and two substrates were compared: peat:perlite (70/30 W/w) and sand:coir (50:50 W/w). This technique guarantees high levels of production and low contamination by avoiding the use of polluted urban soils which are often common in slum areas. Macro and microelements in tomato fruits were analysed by ICP-MS. Results showed low concentrations of heavy metals (Cd, Sr, As, Cr, Mo) with all heavy metals under the levels set by European Community laws. Lack of food safety in slums is more closely linked with malnutrition than starvation, so it is also important to understand mineral availability (both micro and macro) and nutritional significance for health. For this reason micro and macro elements (K, Ca, Na, Fe, Mn, Mg, Cu, Zn) were analysed in the harvested fruits. This study showed that HS is a valid method to grow vegetables in urban areas to improve food security in Middle East cities.

1. Introduction

Food security is a central theme of the new millennium and it must be faced at national and international levels, taking into consideration the multidisciplinary nature of the field which involves socio-cultural, political and environmental, as well as agronomic and economic aspects (Deaton and Paxson, 1998). From an economic and environmental point of view, food security is defined as a situation in which people have safe and appropriate food with nutritional requirements, for an active and healthy life (WFS - Plan of action, 1996). Food security is based on three pillars: food availability, food access, and food uses as reported in the FAO guidelines (Matushke, 2009).

Food security is a priority in all developing countries, in particular in the urban areas of Africa with the Egyptian situation and the conditions of its capital city, Cairo, being critical. Despite the underestimation of risks, the population of the slum areas is affected by malnutrition. In 2005 the Egyptian Demographic and Health Survey stated that 18% of Egyptian youth and 16% of the residents of urban areas were affected by malnutrition. In a city like Cairo, the improvement of agricultural hydroponic systems might be a possible solution to this problem, in particular for informal urban settlements. The development of an efficient and productive growing protocol such as simplified hydroponic systems could be used to grow horticultural produce and increase the availability of natural and safe food for the poorest classes of the population (Dresher, 2004). Food insecurity is a global problem because it is caused by growth of the demand by the world population for secondary food like meat, making, basic resources, such as vegetables and cereals, less available for the poor parts of population (Godfray *et al*., 2010).

The world's urban population is expected to double in the next 30 years, meaning there will be a growing number of urban poor people. According to the United Nations Human Settlement Programme (UN-HABITAT), urban population expansion will be more pronounced in developing countries as a result

(*) Corresponding author: andrea.giro@unimi.it
Received for publication 19 October 2015
Accepted for publication 28 January 2016

of high birth rates and immigration from rural areas as people flock to cities in search of food, employment and security. Population growth will lead to an increase in urban slum areas, with high levels of unemployment, lack of food safety, and malnutrition. The increase of urbanization in developing countries enhances food insecurity of large cities. The poorest people move from rural to urban areas in order to improve their life with hopes of finding a job. Unfortunately, in many cases people do not find employment and are obliged to live in the slum areas, increasing the food demand. For this reason, they become more vulnerable in their new position as citizens and consumers. By 2030, it is estimated that approximately 800 million people in developing countries will live in big cities, instead of in rural areas. This prospective is shocking for many nations, in particular for the social and economic relationships among citizens; this phenomenon is unescapable due to economic development and it could create a negative impact on the food security of populations of cities and, in particular, megacities (Cohen and Garrett, 2010) (Fig. 1).

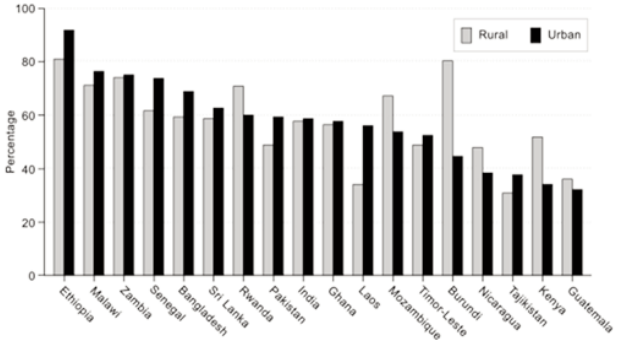

Fig. 1 - Rural and urban index of food energy. International Food Policy Research Institute (IFPRI).

In order to counteract the reduction of food availability in African cities, urban people started growing vegetables in urban and peri-urban areas. Studies showed a considerable degree of self-sufficiency in the production of fresh vegetables, poultry production and the raising of other animals in many large cities of developing countries (Armar-Klemesu, 2000). For example, Dakar produces 60% of its vegetable consumption and produces poultry for 65-70% of the national demand (Mubvami and Mushamba, 2004). Accra produces 90% of the fresh vegetables consumed in the city (Cencosad, 1994; Maxwell *et al*., 2000). In Dar es Salaam, more than 90% of leafy vegetables in the markets come from the open spaces adjacent to the city or in garden houses (Lee-Smith, 2010). Urban agriculture improves the nutritional state of vulnerable communities as evidenced by studies carried out by Mwangi (1995) in Nairobi. However, one of the main problems of urban agriculture is the competition for spaces within the city itself between people, vehicles, and animals. Another important issue is the agricultural knowledge of the people. Many of them do not know how to grow plants, therefore very simple growing systems must be developed. Simplified hydroponic (SH) systems allow cost-effective agricultural production in confined spaces, such as internal urban spaces, particularly in the cities of developing areas (Seikh, 2006). The SH technique allows higher yield, safer food, and at lower costs. The costs of the entire SH system are fundamental for sustainability of the production of goods; for this reason local materials, best if recycled, were used to construct low budget hydroponic systems.

Horticulture works well in urban and peri-urban zones because it is highly labour-intensive, involving perishable products and short-cycle, productive, high-value crops, which require less land and water per unit of product than other food crops. Hydroponic systems are growing techniques that do not use soil, making them suitable in urban areas to cultivate horticultural commodities (Seikh, 2006). It is a relatively young technique used in commercial fields over the past 40 years (Grewal *et al*., 2011). The potential of SH is underestimated: it can be a solution for marginal areas, in developing countries with malnutrition problems. SH technique has the objective of reducing the costs of traditional hydroponic techniques, thus making it available and easy to manage for the poor and people without agriculture skills living in slums, like Al-Quarafa.

For the aims of this study, we used local materials to construct SH greenhouses in order to diminish the cost and to make the technique reproducible for the local population. One of the main costs of SH is the substrate, so it is important to understand which substrate is best in which environment. Standardisation of an efficient protocol of SH is important in order to cultivate horticultural products within the urban context (Santos and Ocampo, 2005). Nowadays, many examples of hydroponics are available in developing countries but they are not usually well set up and tend to be expensive. Hence, the new challenge is represented by the inception

and enhancement of a hydroponic system sustainable in all aspects, from production to management.

2. Materials and Methods

Simplified hydroponic systems and plant cultivation

Boxes made of wood (100x50x20 cm) ensure the best conditions for plant growth and for transportation which are fundamental aspects for the socio-environmental context (Iwasa and Roughgarden, 1984). Tomato plants (*Solanum lycopersicum* L.) require a minimum depth of 20-30 cm for root system development (Pardossi *et al*., 2005). For the present study, plant density was 3 plants/m^2. Individual modules of locally manufactured palm wood boxes (50x50x25 cm) were used to reduce the cost of materials, planting one tomato plant per box. The ratio between the surface and the plant was chosen following the standard for tomato crops in open field. All boxes were plastic-coated with a black polyethylene film in order to maintain the moisture of the substrate, preventing excessive evaporation (Hassan-Wassef, 2004). During the summer season, high temperatures in Cairo cause rapid water evaporation, therefore to reduce this affect, the cultivation boxes were covered with white mulching. Evaluation of substrate performance was carried out by comparing the production of 24 tomato plants. For each substrate, plant growth and fruit quality were determined.

Substrates comparison

Perlite, peat or coconut fibre, and sand were compared. The substrates were mixed and the final composition was 50% sand + 50% coir (S+C) and 70% peat + 30% perlite (P+P). Three steps followed: fertilization and irrigation up to saturation, mulching and planting, and covering with net-shading to reduce sun intensity.

The fertigation strategy used was based on an initial fertilization with 25 g of 20-20-20 NPK fertilizer containing Fe 5 g/kg, Zn 5 g/kg, and Mg 1 g/kg during the preparation of substrate with the addition of 1 g microelements (Table 1). The peat used had the following characteristics: pH 3.5; EC 10 mS/m; hydric retention 70% vol. Peat pH was adjusted to 5.5 by adding 200 g $CaCO_3$. The simplified weekly fertigation schedule provided irrigation with 20 L/week of water containing 3 g $Ca(NO_3)_2$ for each plant. Nitrogen was provided as 13.7% N-NO_3 and 1.5% N-NH4, with a total of 15.2%. The addition of 25 g NPK and 1 g of micronutrients was carried monthly for each box.

Table 1 - EU Heavy metal security limits (EC 420/2011)

Elements	Concentration
Cr	0.050 mg/kg
Pb	0.100 mg/kg
Mo	0.025 mg/kg
As	3.500 mg/kg
Cd	0.050 mg/kg

Assessment of tomato growth and fruit quality

Plant growth was evaluated monthly and the following parameters were recorded: height, time of flowering, and number of flowers for plants in the different substrate conditions. Fruit quality evaluations were carried out on dried fruits and comprised the determination of macro (K, Na, Ca) and microelements, which are important for a balanced human diet. The concentrations of heavy metals (Cd, Pb, Sr, Cr, Mo, As), as possible contaminants, were also measured. Analytical determinations were carried out using an ICP-MS (Inductivity Coupled Plasma-Mass Spectroscopy). To better understand the potential of the cultivation technique related to the lack of food safety in the slum community, we also analysed the quality of tomatoes bought in local markets of the cemetery area of Al-Quarafa. To evaluate the quality, comparisons were made with standard dried tomato analysed by the FDA (Food and Drug Administration). Dry materials were ground and digested with nitric acid (7:0.1). Mineral compounds (Fe, Mn, Zn, and Cu) were measured using inductively coupled plasma mass spectrometry (ICP-MS). Values were reported as means and standard errors (n=3). Data were subjected to ANOVA analysis and differences among means were determined using Bonferroni's post-test.

3. Results

Plants growth and productivity

Plant growth was monitored in sand and coir or peat and perlite in the two growth containers during cultivation. Higher values were found in plants grown in the S+C substrate in both palm boxes (Fig. 2A) and woody boxes (Fig. 2B). However, statistical differ-

ences were found only in woody boxes. In the S+C substrate the plants reached an average height of 50 cm after three months. In the P+P substrate the plants ranged from 35 to 38 cm. In June, in both substrates, plants reached a plateau (Fig. 2).

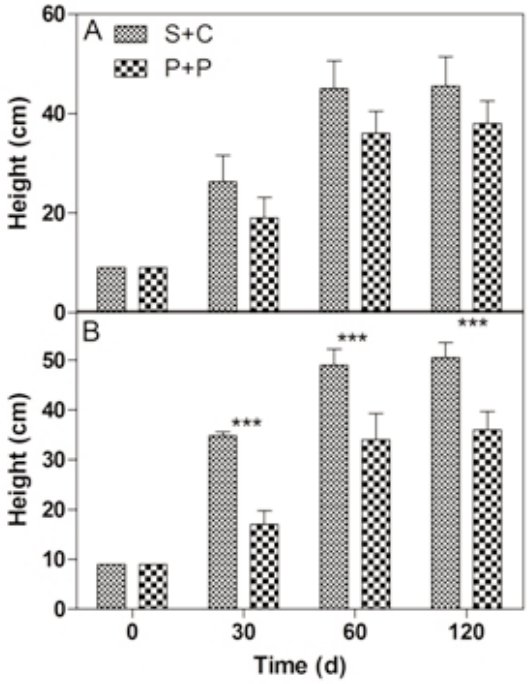

Fig. 2 - Plant height of tomato plants grown in palm boxes (A) or woody boxes (B). Values are means with standard errors. Data were subjected to two-way ANOVA. Differences among means were determined using Bonferroni's post-test.

Food quality, nutrients and heavy metals content

The results revealed that simplified hydroponics guarantee the safety of food produced, even in heavy metals-polluted environments. The main mineral nutrient contents were determined in ripe tomato fruits and compared with those sold in the local markets.

The sodium (Na) content was lower in fruits harvested from plants grown in the S+C substrate (1.7 mg/kg DW) compared with the other samples. The fruits purchased from local markets had a higher Na amount: 7.5 mg/kg DW on average (Fig. 3A). The magnesium (Mg) content in fruits grown in SH was similar without significant differences between the two substrates and ranged from 2.7 to 3.4 mg/kg DW (Fig. 3A). In fruits obtained from local markets, the Mg content was almost double (Fig. 3A). Analogous results were observed for potassium (K) and calcium (Ca). The K in fruits harvested from plants grown in the two different substrates ranged from 65.5 to 68.0 mg/kg DW, while for fruits purchased from the markets the values were higher, 110.0 mg/kg DW on average (Fig. 3). The Ca content was similar to Mg, double the content was found in the commercial tomato fruits compared with those harvested from the SH system (Fig. 3A). Considering the heavy metals microelements, different concentrations were found in the tomato fruits. Copper (Cu) in the analysed fruits ranged from 0.6 to 0.8 mg/100 g DW. The highest values were found in the P+P substrate (Fig. 3). Manganese (Mn) was similar in distribution in the different treatments, but the concentration was higher: from 0.7 to 4.4. mg/100 g DW. The highest value was found in the fruits harvested from plants grown in the P+P substrate (Fig. 3). On the contrary, Zn content was higher in the SH cultivated fruits compared with those obtained from local markets. The values ranged from 1.3 to 2.0 mg/100 g DW. Iron (Fe) was

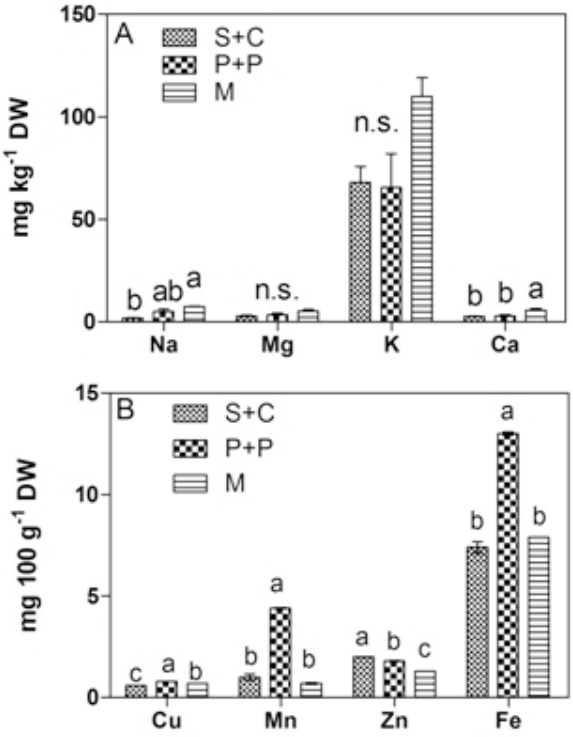

Fig. 3 - Nutritional elements expressed as mg/100 g dried weight. A) Na, Mg, K and Ca. B) Cu, Mn, Zn and Fe in tomato fruits harvested from plants grown in sand and coir (S+C), peat and perlite (P+P) or purchased at the local market (M Values are means with standard errors. Data were subjected to two-way ANOVA analysis and differences among means were determined using Bonferroni's post-test. Different letters indicate statistical differences for <0.05. The heavy metals in fruits were under the safety thresholds established by the European Union.

higher in fruits obtained from plants grown in the P+P substrates and the mean was 13.0 mg/100 g DW. Half the amount was found in fruits harvested from the S+C grown plants and purchased from local markets (Fig. 3).

Heavy metals were found in trace amounts and the values were below those set by EU regulations. The As content was higher in local market fruits (6.03 µg/kg DW), while in the SH system the As content was 0.63 µg/kg DW in fruits harvested from plants grown on the S+C substrate and 2.58 µg/kg DW in those obtained from plants grown on P+P substrate. Strontium (Sr) in the SH system ranged from 17.97 to 20.7 µg/kg DW, while in tomatoes purchased from the local market the concentration was 44.6 µg/kg DW on average (Fig. 4). Molybdenum (Mo) was 10-fold higher in fruits harvested from plants grown in the P+P substrate compared with S+C and local market tomato samples. Cadmium (Cd) was higher in fruits sold in local markets compared with those obtained from the SH system. The lowest value, 1.6 µg/kg DW, was found in fruits harvested from plants grown on the P+P substrate. Analogous results were observed for lead (Pb); the highest value found in fruits purchased from the market was 28.97 µg/kg DW (Fig. 4). On the contrary, higher Cr values were found in the fruits harvested from the SH system (21.03-45.67 µg/kg DW) compared with 16.83 µg/kg DW measured in the fruits purchased at the market (Fig. 4).

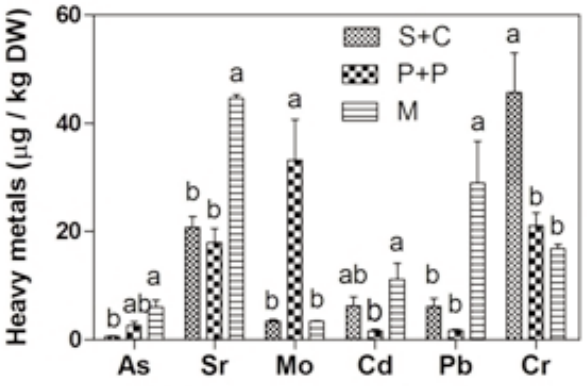

Fig. 4 - Heavy metals expressed as µg/kg DW determined in tomato fruits harvested from plants grown in sand and coir (S+C), peat+perlite (P+P) or purchased from the local market (M). Values are means with standard errors. Data were subjected to two-way ANOVA analysis and differences among means were determined using Bonferroni's post-test. Different letters indicate statistical differences for <0.05.

4. Discussion and Conclusions

The cultivation of vegetables in highly polluted environments where there is poor soil quality can only be achieved using soilless systems. Most people living in the slum areas do not have skills or knowledge about agricultural practises, therefore urban agricultural systems must be simple and easy to manage. Unfortunately, high density urban and peri-urban areas can also be subjected to heavy metal pollution. Contamination can also come from airborne trace elements such as Cd and Mo (Francini *et al*., 2010) that are deposited on the surface of fruits. Heavy metal accumulation in vegetables is closely correlated with the pollution level of the area (Antisari *et al*., 2015). SH is a good strategy to improve food security for urban slum populations and, in particular, it is linked to an increase of food availability (Orsini *et al*., 2013). However, the food situation could also improve through direct sale of the products if the cultivated surface were enough to have a surplus of fresh produce. For these reasons it is important when choosing the cultivar to consider the most profitable ones in order to improve saleability of the products. The SH technique makes it possible to cultivate safe vegetables, in small spaces, and with low-tech instruments.

The results of the current study reveal that local materials for substrate and palm boxes were a good compromise between cost and production capability. The community of Al-Quarafa follows a typical Mediterranean diet based on cereals and legumes, however they do not consume fresh vegetables because they are very expensive and perishable (Arenas and Vavrina, 2002; Hamza and Mason, 2004). Thus, the application of SH represents an interesting method to combat food insecurity in Al-Quarafa, a symbol of all Cairo slums. Moreover, the use of the substrates in the study avoided the accumulation of heavy metals in vegetables and allowed enrichment of some of the micronutrients that improve human health.

SH have been used in Africa for 10 years to cultivate fresh products in urban and peri-urban settlements. For example in Dakar, in 1999 a FAO pilot project for family farming employed SH. The Dakar models, however, were adapted to tropical temperature and climate. The substrates were 40% peanut shells, 40% rice husks, and 20% perlite, suitable for leafy vegetable production because they are harvested at a young stage and do not have vertical growth.

Unfortunately, they are not able to support the growth of plants such as tomatoes. In fact, tomatoes prefer more stable substrates, from a mechanical point of view, such as peat/perlite or coir/sand (Esposito, 2010). Also the depth (14-20 cm) of the recycled-pallet boxes used in Dakar was less than that required by cultivated varieties that need greater volume for adequate root development to favour production (Ghehsareh *et al*., 2011). The irrigation schedule in the Dakar project provided 2 L/m^2 two to three times a day (Ghehsareh *et al*., 2011). The nutritional elements were recollected in a small container under the boxes. This daily schedule means that plants must be managed three times a day and this is one of the differences between the Dakar project and the method applied in the current study tested in Cairo in which an irrigation schedule was followed of once a week, thanks to the water capacity (WC) of the peat and coir. The combination of 50% coir and 50% sand provided the best growth performance for two reasons: 1) because using a weekly irrigation schedule the coir had greater WC than peat (Abad, 2002), and 2) because the weight of sand simplified the growth of the vegetative part of the plants, offering mechanical stability.

It is also important to consider the pH of the substrate used: the combination between coir and sand naturally had a sub acid pH (Arenas and Vavrina, 2002), with is the naturally ideal pH for tomato growth. This aspect favoured the availability of the micro and macro nutrients (Ghehsareh *et al*., 2011). From a productive point of view, the tomato plants produced on average 0.4-0.5 kg per plant in the current investigation; the best results were achieved with tomatoes grown in sand/coir. Considering two plants per box, these values translate to a productive capability of 0.8-1 kg/m^2 of tomatoes on average. These productivity ranges consider the stressed conditions of the climate in Cairo which allows two growing cycles per year (September to November, March to June).

In terms of nutrition, considering the nutritional level of macro and micro elements of tomatoes analysed by the FDA, the tomatoes grown in SH in the current study had similar nutritional levels and sometimes better than the tomatoes the FDA considered as standard.

Social agriculture and future strategy

This SH growing system has been developed for poor urban communities with very limited agricultural knowledge. These cultivation systems are suitable for urban and social agriculture as reported in FAO guidelines. Moreover, for production in highly populated areas, SH systems also play a social role as they can be meeting points for the families within the community. Therefore, these growing systems should not be considered only as vegetable production tools. These systems must be easy to manage especially for water and nutrients supply. The management of the cultivation is often carried out by women and children, who are the most susceptible to food insecurity. In slum areas like Al-Quarafa, people are depressed, marginalized and unemployed. Hence, the growing areas improve food production, as well as the physic and psychological health of the inhabitants. Collaborations among the people living the community improve relationships between families and create opportunities to establish networks. These networks are essential to sharing information on crop cultivation in SH systems and to organising selling points in local market. In the future, the "Cairo model" of the SH system may be exported to other depressed urban areas in Africa and in the Middle East. This new, low-cost and low-tech growing system for vegetable production provides a great opportunity for poor populations who want to redeem themselves within society.

Acknowledgements

This study was logistically supported by NGO "LiveinSlums" and financially supported by Municipality of Milan.

References

ABAD M., 2002 - *Physico-chemical and chemical properties of some coconuts dust for use as a peat substrate for containerised ornamental plants*. - Bio-resource Technology, 82: 241-245.

ANTISARI L.V., CARBONE S., GATTI A., VIANELLO G., NANNIPIERI P., 2015 - *Uptake and translocation of metals and nutrients in tomato grown in soil polluted with metal oxide (CeO_2, Fe_3O_4, SnO_2, TiO_2) or metallic (Ag, Co, Ni) engineered nanoparticles.*- Environ. Sci. Pollut. Res., 22(3): 1841-1853.

ARENAS M., VAVRINA C.S., 2002 - *Coir as an alternative to peat in media for tomato trasplant production*. - HortScience, 37(2): 309-312.

ARMAR-KLEMESU M., 2000 - *Urban agriculture and food security, nutrition and health*, pp 99-118. In: BAKKER N., DUBBELING M., GUNDEL S., SABEL-KOSCHELLA S.,

DE ZEEUW H., (eds.*) Growing cities, growing food: Urban agriculture on the policy agenda. A reader on urban agriculture*. GTZ/DSE, Germany.

CENCOSAD, 1994 - *Urban market gardens in Accra.* - Centre for Community Studies, Action and Development and the Mega Cities Project, Accra, Ghana.

COHEN M.J., GARRETT J.L., 2010 - *The food price crisis and urban food (in) security*. - Environment & Urbanization, 22(2): 467-482.

DEATON A., PAXSON C., 1998 - *Economies of scale, household size, and the demand for food.* - Journal of Political Economy, 106(5): 897-930.

DRESHER A.W., 2004 - *Food for cities: Urban agriculture in developing countries*. - Acta Horticulturae, 643: 227-231.

ESPOSITO T., 2010 - *Agriculure urbaine et periurbaine pour la securite alimantaire en Afrique de l'Ouest. Le cas des micro-jardin dans la municipalite de Dakar*. - PhD thesis, Facoltà di Agraria, Università di Milano, Italy, pp. 141-147.

FRANCINI A., NALI C., LORENZINI G., LOPPI S., 2010 - *Leaves of* Lolium multiflorum *as indicators of airborne trace element distribution in Central Italy*. - Int. J. Environment and Health, 4(2-3): 151-165.

GHEHSAREH A.M., SAMADI N., BORJI H., 2013 - *Comparison of date-palm wastes and perlite as growth substrates on some tomato growing indexes*. - African J. of Biotechnology, 10(24): 4871-4878.

GODFRAY H.C., BEDDINGTON J.R., CRUTE I.R., HADDAD L., LAWRENCE D., MUIR J.F., TOULMIN C., 2010 - *Food security: the challenge of feeding 9 billion people*. - Science, 327(5967): 812-818.

GREWAL H.S., MAHESHWARI B., PARKS S.E., 2011 - *Water and nutrient use efficiency of low-cost hydroponic greenhouse for cucumber crop: An Australian case study* - Agricultural Water Management, 98: 841-846.

HAMZA W., MASON S., 2004 - *Water availability and food security challenges in Egypt.* - International Forum on Food Security Under Water Scarcity in the Middle East: Problems and Solutions. Como, Italy, 24-27 Nov, pp. 12.

HASSAN-WASSEF H., 2004 - *Food habits of the Egyptians: newly emerging trends.* - East Mediterr Health J, 10(6): 898-915.

IWASA Y., ROUGHGARDEN R., 1984 - *Shoot/root balance of plants: optimal growth of a system with many vegetative organs.* - Theoretical Population Biology, (25): 78-105.

LEE-SMITH D., 2010 - *Cities feeding people: an update on urban agriculture in equatorial Africa*. - Environment and Urbanization, 22(2): 483-499.

MATUSHKE I., 2009 - *Rapid urbanization and food security: Using food density maps to identify future food security hotspots.* - FAO contributed paper for International Association of Agriculture Economist Conference, Beijing, China, August 16-22, pp. 15.

MAXWELL D., LEVIN C., ARMAR-KLEMESU M., RUEL M.T., MORRIS S.S., AHIADEKE C., 2000 - *Urban livelihoods and food and nutrition security in Greater Accra*. - The International Food Policy Research Institute Research Report 112. International Food Policy Research Institute, Washington, D.C., USA.

MUBVAMI T., MUSHAMBA S., 2004 - *Mainstreaming gender in urban agriculture: Application of gender analysis tools: A case study of Musikavanhu Project, Harare. Municipal Development Partnership, Harare Zimbabwe*. - Women Feeding Cities, Accra, Ghana, Sept. 20-23, pp. 42-43.

MWANGI A.M., 1995 - *The role of urban agriculture for food security in low income areas in Nairobi* - Report 54/1995, Ministry of planning and national development, Nairobi, and African Studies Centre, Leiden, Kenya, pp. 42-44.

ORSINI F., KAHANE R., NONO-WOMDIM R., GIANQUINTO G., 2013 - *Urban agriculture in the developing world: a review*. - Agron. Sustain. Dev., 33: 695-720.

PARDOSSI A., MALORGIO F., INCROCCI L., TOGNONI F., 2005 - *Hydroponic technologies for greenhouse crops*, pp. 360-378. - In: RAMDANE D. (ed.) *Crops: quality, growth and biotechnology.* - WFL Publisher Science & Tecnology, Helsinki, pp. 1356.

SANTOS P.J., OCAMPO E.T., 2005 - *Snap hydroponics: Development & potential for urban vegetable production.* - Philippine Journal of Crop Science, 30(2): 3-11.

SEIKH B.A., 2006 - *Hydroponics: key to sustain agriculture in water stressed and urban environment*. - Pak. J. Agric., Agril. Eng., Vet. Sci., 22: 53-57.

Adv. Hort. Sci., 2016 30(1): 31-37

Characterization and evaluation of *Berberis microphylla* G. Forst pollen grains

S. Radice (*), M. Arena
CONICET, Facultad de Agronomía y Ciencias Agroalimentarias, Universidad de Morón, Machado 914, B17108EOH Morón, Buenos Aires, Argentina.

Key words: barberry, germination, Patagonia, size, viability.

Abstract: *Berberis microphylla,* commonly known as "calafate", is a non-timber forest product native from Patagonia, and its berries possess highlighted nutraceutical value. The objective of this research was to describe the morphology and anatomy of pollen grains of *Berberis microphylla* G. Forst genotypes growing spontaneously on the island of Tierra del Fuego (Argentina), and evaluate their vitality and germination. Pollen grain diameter varied from 40 to 47.26 µm, the pollen grains of 124 and 201 genotypes being significantly smaller than the others. Vitality measured by DAPI methodology was also variable among genotypes, although always about 50%. *In vitro* germination of pollen grains measured one day after the flowers were collected was very high for some genotypes (near 80%), and then decreased after 21 days of storage, except for genotype 123 whose germination value increased from 44.34 to 69%. The significant variability found in pollen performance (size, viability and germination) among *B. microphylla* genotypes from a natural population could be interpreted as an enhanced survival strategy to maximize reproduction fitness, with a marked capacity of response to environmental changes. High viable pollen frequency together with germination percentages observed in all the genotypes tested could indicate a good fertilization process. The correlation observed between size and germination percentage could be used as markers of pollen grain performance, paving the way for possible *B. microphylla* breeding.

1. Introduction

In spite of the well-known importance of wild flora as a source of food and medicinal substances, more studies on the diversity and agronomic value of these plant species are still needed (Arena and Vater, 2005). Areas with indigenous flora offer non-domesticated plants (Monge *et al.*, 2000), like *Berberis* genus in Patagonia (Orsi, 1984) for these purposes. *Berberidaceae* in the widest sense is a small family, consisting of 10 to 12 genera and about 600 species, with as many as 500 of these belonging to *Berberis* L., widely distributed in both the Old World and New World (Nowicke and Skvarla, 1981). In Patagonia, *Berberis* genus is well represented by 16 species of native shrubs and they are distributed from Neuquén to Tierra del Fuego (Arena and Curvetto, 2008). However, according to a later classification of the genus (Landrum, 1999), the number of species is less than previous studies cited by Orsi (1984), as Landrum groups the species *B. buxifolia*, *B. microphylla* and *B. heterophylla* under *B. microphylla* G. Forst, postulating that the differences among them may fluctuate to retain its range of species.

In particular, *B. microphylla* (ex *B. buxifolia* Lam.), commonly known as "calafate", is an evergreen shrub that is present throughout the region mentioned, prevalent on the island of Tierra del Fuego over other species of *Berberis*. *B. microphylla* has growing economic potential due to the production of fruits as a non-timber forest product (Tacón Clavaín, 2004). In fact, its dark blue berries are consumed fresh, as jams and preserves, and are used for the production of soft drinks and ice cream. Moreover, the fruits have a high content of phenols and antioxidants (Arena and Curvetto, 2008; Arena *et al.*, 2012). Some characteristics of its phenological phases (Arena *et al.*, 2013 a; Arena and Radice, 2014), fruit composition and production (Arena and Curvetto, 2008; Arena *et al.*, 2003; 2011; 2013 b) have already been studied in natural populations of this species.

Characterization of pollen grains is an important step for programs of genetic resource conservation and improvement, complementing basic studies of biological data that characterize genotypes (de Castro Nunes *et al.*, 2012). Selection on male game-

(*) Corresponding author: siradice@yahoo.com
Received for publication 31 October 2015
Accepted for publication 4 February 2016

tophytes (pollen) to alter the genetic constitution of the subsequent sporophytic generation has been suggested as an interesting tool in plant breeding programs (Hormaza and Herrero, 1992). Pollen competitive ability describes the reproductive success of a pollen grain and can therefore be considered as equivalent to pollen fitness (Sari-Gorla and Frova, 2005). The variability in pollen performance (size, viability and germination) among genotypes of a natural population could be interpreted as a survival strategy to maximize reproduction fitness (Tejaswini, 2002), while enabling a capacity of response to environmental changes (Hedly *et al.*, 2005). Nevertheless, aspects related to fertility and reproductive organs have not yet been studied on *Berberis microphylla.*

The objective of this research was to describe the morphology and anatomy of pollen grains of *Berberis microphylla* G. Forst genotypes growing spontaneously on the island of Tierra del Fuego (Argentina), and evaluate their vitality and germination.

2. Materials and Methods

Plant material

Flowers (*n=20*) in phase E (before anthesis according to Arena *et al*., 2011) were collected from each *B. microphylla* genotype grown near Ushuaia city, Tierra del Fuego (54° 48' SL, 68° 19' WL and 30 m asl) (Table 1), in October 2013. The flowers were immediately placed in Petri dishes with wet paper at 5°C for viability and germination studies.

Table 1 - *Berberis microphylla* genotype number and its satelital position in Tierra del Fuego (Argentina)

Genotype number	SL	WL
107	54 49 43 0	68 19 01 7
108	54 49 42 9	68 19 02 1
111	54 49 43 5	68 19 00 1
122	54 49 40 9	68 19 04 1
123	54 49 42 4	68 19 07 1
124	54 49 42 8	68 19 04 2
125	54 49 46 1	68 19 00 6
126	54 49 45 4	68 18 58 7
177	54 49 42 9	68 19 27 9
201	54 49 50 7	68 19 21 7
202	54 49 51 2	68 19 20 1

Pollen grain description and size

Equatorial and polar diameters of the pollen grains (*n=50*, randomly selected) were measured for each studied genotype using a Leica DM 2500 microscope. The average of the two parameters for each pollen grain was then calculated.

Light microscopy

Button flowers were dehydrated in an ethanol series and embedded in Spurr's resin. Thin sections (75-90 nm thick) were stained with uranyl acetate and lead citrate.

Scanning electron microscopy

Button flowers were dehydrated in an ethanol series and a critical point drying technique was employed. Samples were sputter coated with 20 nm gold and observed with a Philips XL 30 SEM.

Transmission electron microscopy

Anthers were pre-fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and then post-fixed in OsO_4 at 2°C in the same buffer for 3 h. They were then dehydrated in ethanol series and embedded in Spurr's resin. Thin sections (75-90 nm thick) were made on a Sorval ultramicrotome, stained with uranyl acetate and lead citrate (O'Brien and Mc Cully, 1981). Sections were observed with a Jeol-Jem 1200 EXII TEM at 85.0 kv.

Pollen grain viability

Pollen grains were hydrated with sucrose solution (15%) and treated with fluorescein diacetate (10%) and propidium iodine (2%) (Greissl, 1989). The number of viable and not viable pollen grains was recorded under optic microscope, with a minimum of 300 pollen grains per genotype.

Pollen grain germination

Pollen grains were put on micro drops of a saline solution composed of 2 x 10^{-3} M H_3BO_3 and 6 x10^{-3} M Ca $(NO_3)_2$ added with sucrose 30% (Dafni, 1992). Micro drops were placed on the inside of the lid of a petri dish in which 3 ml of water were added in the base to create a humid chamber. Incubation was at 21±2°C. The number of germinated and aborted pollen grains was recorded under optic microscope 24 h after the test started and performed with anthers conserved for 1, 10 and 21 days at 5°C.

Data analysis

Measurements were analyzed by ANOVA and Tukey's test and chi-square test was employed to evaluate pollen vitality.

3. Results

Pollen grain description and size

Flowers collected in phase E had five stamens; the anthers were not yet dehiscent (Fig. 1A) although the pollen grains were already mature (Fig. 1B, 2A-B). A mature pollen grain is formed of a vegetative cell with a dense cytoplasm with numerous starch grains (Fig. 1B). *B. microphylla* pollen grains are spherical with a psilate punctate surface interrupted by mild cracks (Fig 2. A-B), resembling a tennis ball.

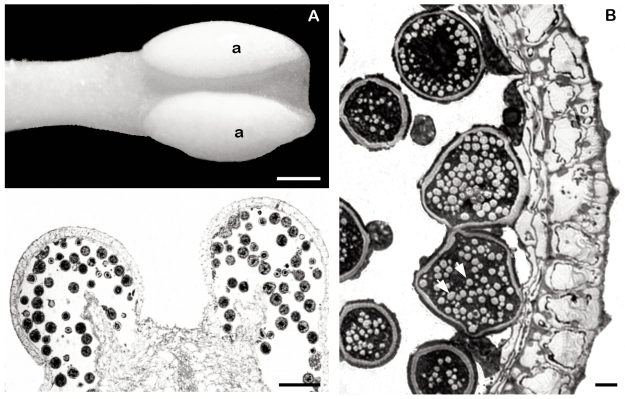

Fig. 1 - Androecium and pollen grain of *B. microphylla* flower in phase E. A) view of androecium with anther (a) no dehiscent; B-C) microphotograph of cross-section of anther and mature pollen grain with starch grains (arrowhead). Bars: A = 1mm; B = 10 µm; C = 100 µm.

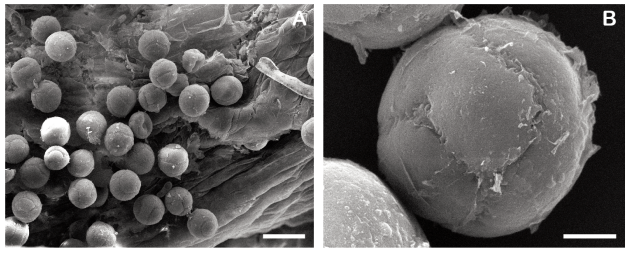

Fig. 2 - SEM micrograph of mature pollen grain of *B. microphylla*. A) view of microsporangium with pollen grain; B) detail of a pollen grain. Bars: A = 10,000 nm; B = 50 µm.

The pollen wall is formed by an exine and intine of considerable thickness (Fig. 3A). Transmission electron micrographs make it possible to identify an exine with two different layers, the ektexine and the endexine (Fig. 3A). Ektexine is nearly amorphous and not organized into typical foot layer, columellae, and tectum units. Conversely, ektexine appears as an external irregular cover with channels and small enclosed areas which are electron translucent (Fig. 3). Endexine has greater electro-density than ektexine. Immediately below this, the intine is present, at least four times thinner than the exine and with a

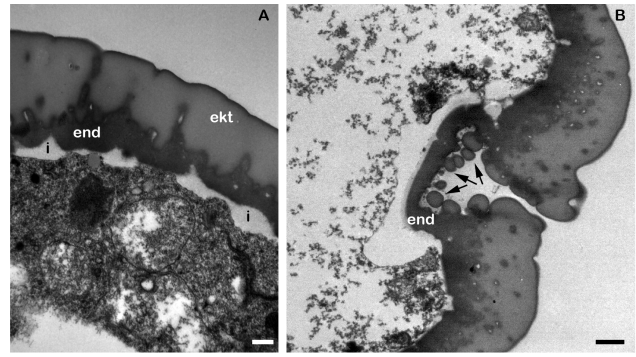

Fig. 3 - TEM micrograph of pollen grain wall of *B. microphylla.* A) Detail of different parts of exine: endexine (end), ektexine (ekt) and intine (i); B) Detail of pore zone with ektexine fragmented (arrows). Barras = A-B = 1 µm.

very low electro-density. Pollen grain wall appears different on the apertures or pore zone (Fig. 3B). Ektexine is less structured and is represented by nodules above the endexine.

The pollen grain diameters varied significantly among genotypes, from 40 to 47.26 µm ($p \leq 0.001$). The maximum values were observed for genotype 107, which was significantly higher than genotypes 124, 126, 177, and 201 (Fig. 4). Pollen grains of genotypes 124 and 201 were significantly smaller than genotypes 107, 111, 123 and 202 (Fig. 4).

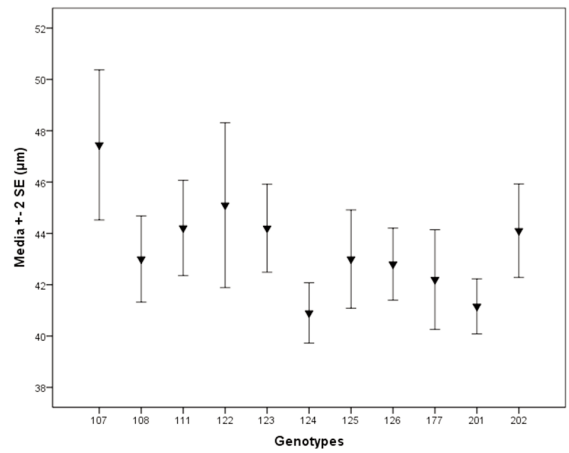

Fig. 4 - Mean diameter of *B. microphylla* pollen grains of the studied genotypes.

Pollen grain viability

Pollen grains stained with fluorescein diacetate and propidium iodine showed very different colors depending on whether they were vital or non-vital.

Vital pollen grains were bright green while non vital ones stained red (Fig. 5). In this latter case, another category was evaluated, the sub-vital pollen grains, those that can germinate but it is uncertain whether they can be efficient in fertilization. Pollen viability of different genotypes gave very different results ($p \leq 0.001$). For genotypes 111, 123, 124 and 202, values of vital pollen grains were above 70%, while for genotype 108 the value was 51.47% (Fig. 6). In coincidence, the 108 genotype shows a 36.80% of sub-vital pollen grains, value significantly greater than that observed for the 111, 123, 124, 126, and 202 genotypes.

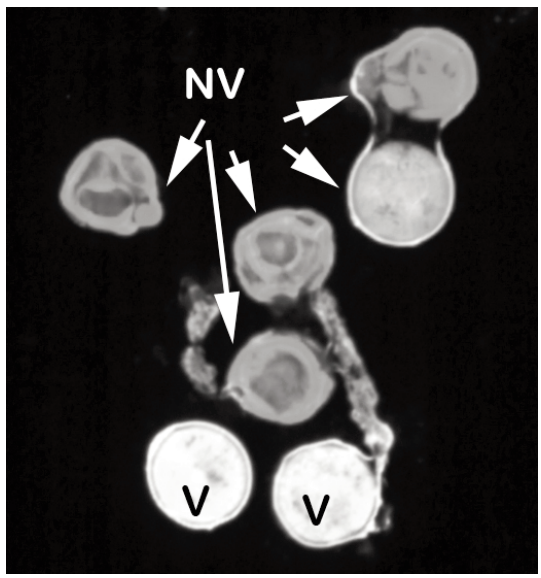

Fig. 5 - Viable (V), and non-viable (NV) pollen grains observed by fluorescence microscopy.

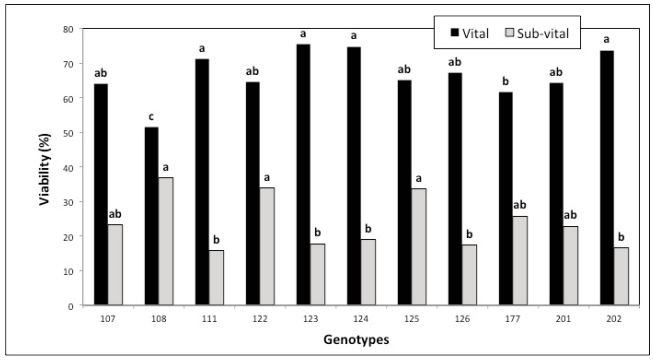

Fig. 6 - Viability of *B. microphylla* pollen grains from the assessed genotypes. Columns with different letters indicate significant differences between genotypes and date assessed (χ^2 $p<0.05$).

Pollen grain germination

The pollen grain germination of *B. microphylla* was significantly different among genotypes ($p \leq 0.001$) and days of conservation ($p \leq 0.001$), and the interaction between the two factors studied ($p \leq 0.001$) was significant (Fig. 7). Germination of the pollen grain was at maximal level after one day of collection in most genotypes, except for genotype 123 which showed a maximum value after 21 days of storage, although without significant differences between 10 and 21 days (Fig. 7). Genotypes 124 and 125 showed percentages of pollen grain germination up to 70%, while genotype 201 presented a maximum value of 87.03% after one day of the collected flowers (Fig. 7). The pollen germination rate remained unchanged among the three tested dates, except for genotypes 122, 126 and 177. In addition, the values were significantly lower after 21 days of conservation for genotypes 122, 126 and 177 (Fig. 7). This decrease in the percentage of pollen germination is in accordance with the percentage of aborted pollen grains (data not shown). ANOVA values were 0.001 for both date and genotype factor and their

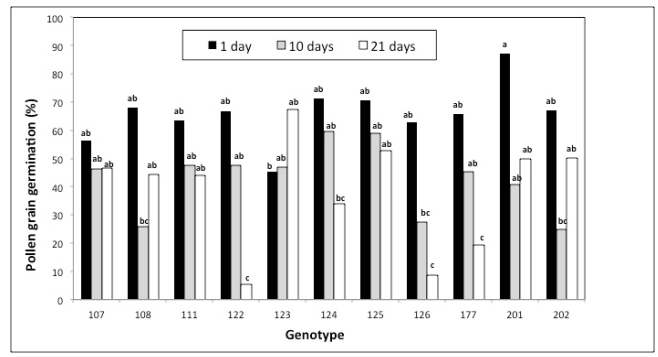

Fig. 7 - Germination percentage of *B. microphylla* pollen grains from the assessed genotypes. Columns with different letters indicate significant differences between genotypes and date assessed (Tukey $p<0.05$).

interaction. Furthermore, the genotypes 108 and 202 showed an increase in the percentage of germinated pollen grains between the second and third test, i.e. the values obtained after 21 days of flower conservation were higher than those obtained with 10 days of storage but these differences were not significant (Fig. 7).

Finally, a significant negative correlation between the pollen grain germination and the pollen grain size was found ($r = -0.252$; $p \leq 0.001$).

4. Discussion and Conclusions

It was observed that flower differentiation on *B. microphylla* started 12 weeks after bud break in coincidence with the end of the first fruit growth phase

(Arena and Radice, 2014). Nevertheless, final development of male gametes occurs the following spring when the flower bud elongates (data not shown). Mature pollen grains can be found in the stage of lower emergence code 59 according to the BBCH scale (Arena *et al*., 2013 a). There are few published studies on the pollen of *Berberis* species. Erdtman (1952) and Heusser (1971) described the pollen grains of the *Berberis* genus and they determined that the pollen grains measured an average of 30 to 65 µm with an exine 2-3 µm thick. Nowicke and Skvarla (1981) emphasized the presence of irregular apertures, a psilate surface and an unstratified exine. In adittion, the exine of *B. micropylla* shows an endexine as a prominent fibrous-granular layer and the ektexine with cavities and channels which suggest the endexine.

Pollen performance traits are often genetically based (Hedly *et al.*, 2005; Hove and Mazer, 2013), however they could be also affected by differences in the nutritive status of the developing pollen grains as well as by the environmental conditions (Hedly *et al.*, 2005). The size of pollen grains is considered to be one indicator of their viability (i.e. germinability and pollen tube growth rate), while the proportion of large pollen grains has been used to estimate pollen performance. Variation in pollen grain size among plants has been documented for several species (Varis *et al.*, 2011). Larger pollen grains are thought to contain more resources for germination and, thus, have greater viability than smaller grains (Dufaÿ *et al.*, 2008). However, in *B. microphylla* a negative correlation between pollen size and germination percentage was observed, as was also found in *Pinus sylvestris* (Varis *et al.*, 2011), as the growth of the pollen tube may be more dependent on pollen storage than pollen size. Good fertilization is directly related to very good pollen viability. It is estimated that the value of viability should be above 70% for fruit production (Urquieta, 2010). On the other hand, pollen grains which exceed 50% viability would be the only one that can be selected for use as male parents (Urquieta, 2010). All genotypes of *B. microphylla* tested presented viability values above 50%, in other words having very good prospects for fruit production. Urquieta (2010) found similar results when pollen grains of *B. bidentata*, *B. darwinii, B. parodii* and *B. trigona* were tested. Pollen grain viability of these species was variable between 59.6 and 74.1%.

It has been found that high percentages of pollen viability are due to high degrees of adaptability of the species to different environmental conditions (Kelly *et al*., 2002). In effect, high frequency of viable pollen reflects the adaptability of the species, since environmental plasticity submitted by pollen allows the genotype a satisfactory performance to different environmental conditions (Paupière *et al.*, 2014). On the other hand, pollen viability was influenced by relative humidity, temperature, atmospheric composition, and oxygen pressure after release from the anthers (Bots and Mariani, 2005), so it is expected that the experimental values obtained for *B. microphylla* are less than true values.

Pollen germination under *in vitro* conditions always produces lower values than those obtained with the viability test (Ontivero *et al.*, 2006). In the present study, this premise is true for genotypes 107, 111, 123, 124, and 202 (Figs. 6 and 7). On the contrary, all other genotypes showed viability values higher than those obtained by germination viability test. Note that after 21 days of harvested flowers, pollen germination values obtained were only 10% lower than viability values for genotypes 108 and 123. These results could be due to the protective effect that antioxidants have on pollen. In effect, it is well known that secondary metabolites produced in the tapetum, such as phenolic compounds, can spread to the pollen and play a role in pollen colour, in the attraction of pollinators, in pollen tube germination, and in protection against abiotic stress of pollen (Paupière *et al.*, 2014). Pollen germination and tube growth is largely due to the presence of flavonols in mature pollen grains (Yistra *et al.*, 1992). Although the content of flavonols was not measured in the anthers of *B. microphylla*, it is well known that flavonols are present in *Berberis* species (Končić *et al.*, 2010). In effect, accumulation patterns of phenolic compounds during fruit growth and ripening in *B. buxifolia* (*B. microphylla*) was studied by Arena *et al*. (2012). It is likely that high values obtained from *in vitro* germination of pollen grains could be explained by the protective effect that these compounds perform.

The variability found in pollen performance (size, viability and germination) among genotypes of the natural population of *B. microphylla,* and its correlations, suggest the existence of pollen competition leading to unequal reproductive success in this species, as was observed for *Camellia sinensis* by Muoki *et al.* (2007).

The significant variability found in pollen performance (size, viability and germination) among *B. microphylla* genotypes from a natural population could be interpreted as a highlighted survival strategy

to maximize reproduction fitness, with a marked capacity of response to environmental changes. High viable pollen frequency, together with germination percentages observed in all the genotypes tested, could indicate a good fertilization process. The correlation observed between size and germination percentage could be used as a marker of pollen grain performance, with these findings representing the first antecedents useful for *B. microphylla* breeding.

Acknowledgements

The authors thank the Prefectura Naval Argentina and Mrs. Isabel Farías for her assistance with the histology. This research was supported by grants PIP 314 subsidized by CONICET.

References

ARENA M.E., CURVETTO N., 2008 - Berberis buxifolia *fruiting: Kinetic growth behavior and evolution of chemical properties during the fruiting period and different growing seasons*. - Scientia Horticulturae, 118(2): 120-127.

ARENA M.E., GIORDANI E., RADICE S., 2011 - *Flowering, fruiting and leaf and seed variability in* Berberis buxifolia*, a Patagonian native fruit species*, pp. 117-136. - MARIN L., and D. KOVAÈ (eds.) *Native species: Identification, conservation and restoration*, Nova Sciencia Publisher Inc., Hauppauge, NY, USA, pp. 176.

ARENA M.E., GIORDANI E., RADICE S., 2013 a - *Phenological growth and development stages of the native Patagonian fruit species* Berberis buxifolia *Lam*. - J. Food, Agric. Environ., 11(3-4): 1323-1327.

ARENA M.E., POSTEMSKY P., CURVETTO N.R., 2012 - *Accumulation patterns of phenolic compounds during fruit growth and ripening of* Berberis buxifolia*, a native Patagonian species*. - New Zealand Journal of Botany, 50(1): 15-28.

ARENA M.E., RADICE S., 2014 - *Shoot growth and development of* Berberis buxifolia *Lam. in Tierra del Fuego (Patagonia)*. - Scientia Horticulturae, 165: 5-12.

ARENA M.E., VATER G., 2005 - *Native and introduced species of small fruits in Austral Patagonia, Tierra del Fuego*, pp. 96-110. - In: DRIS R. (ed.) *Fruits: Growth, nutrition, and quality*. WFL Publisher, Helsinki, Finlandia, pp. 220.

ARENA M.E., VATER G., PERI P., 2003 - *Fruit production of* Berberis buxifolia *Lam. in Tierra del Fuego*. - HortScience, 38(2): 200-202.

ARENA M.E., ZULETA A., DYNER L., CONSTENLA D., CECI M., CURVETTO N.R., 2013 b - Berberis buxifolia *fruit growth and ripening: evolution in carbohydrate and organic acid contents*. - Scientia Horticulturae, 158: 52-58.

BOTS M., MARIANI C., 2005 - *Pollen viability in the field*. - Research Reports No. CGM5.

DAFNI A., 1992 - *Pollination ecology: A practical approach*. - Oxford University Press, Oxford, UK, pp. 250.

DE CASTRO NUNES R., OLIVEIRA BUSTAMANTE F., TECHIO V.H., MITTELMAN A., 2012 - *Morphology and pollen viability of* Lolium multiflorum *Lam.* - Ciência e Agrotecnologia, 36(2): 180-188.

DUFAŸ M., VAUDEY V., DE CAUWER I., TOUZET P., CUGUEN J., ARNAUD J.F., 2008 - *Variation in pollen production and pollen viability in natural populations of gynodioecious* Beta vulgaris *ssp. maritima: evidence for a cost of restoration of male function?* - J. Evol. Biol., 21: 202-212.

ERDTMAN G., 1952 - *Pollen morphology and plant taxonomy*. - GFF, 74(4): 526-527.

GREISSL R., 1989 - *Vitality analysis of monadic and polyadic pollen grains using optical contrast-fluorescence microscopy*. - Scientific and Technical Information, IX(5): 180-184.

HEDLY A., HORMAZA J.I., HERRERO M., 2005 - *Influence of genotype-temperature interaction on pollen performance.* - J. Evol. Biol., 1(8): 1494-1502.

HEUSSER C.J., 1971 - *Pollen and spores of Chile: modern types of the* Pteridophyta*,* Gymnospermae*,* Angiospermae. - University of Arizona Press, Tucson, Arizona, USA, pp. 167.

HORMAZA J.I., HERRERO M., 1992 - *Pollen selection*. - Theoretical and Applied Genetics, 83**:** 663-672.

HOVE A.A., MAZER S.J., 2013 - *Pollen performance in* Clarkia *taxa with contrasting mating systems: Implications for male gametophytic evolution in selfers and outcrossers*. - Plants, 2: 248-278.

KELLY J.K., RASCH A., KALISZ S., 2002 - *A method to estimate pollen viability from pollen size variation*. - Am. J. Bot., 89(6): 1021-1023.

KONČIĆ M.Z., KREMER D., KARLOVIĆ K., KOSALEC I., 2010 - *Evaluation of antioxidant activities and phenolic content of* Berberis vulgaris *L. and* Berberis croatica *Horvat*. - Food and chemical toxicology**,** 48(8): 2176-2180.

LANDRUM L.R., 1999 - *Revision of* Berberis *(Berberidaceae) in Chile and adjacent Southern Argentina.* - Ann. Missouri Bot. Gard., 86(4): 793-834.

MONGE A., CHORGADE M., ERHARDT P.W., GENELLIN C.R., KOGA N., LINDBERG P., PERUN T.J., TOPLISS J.G., TRIVEDI B.K., WERMUTH C.G., 2000 - *Medicinal chemistry in the development of societies: biodiversity and natural products*. - Eur. J. Med. Chem.**,** 35(12)*:* 1121-1125.

MUOKI R.C., WACHIRA F.N., PATHAK R., KAMUNYA S.M., 2007 - *Potential male gametophyte competition among* Camellia sinensis *genotypes in isolated biclonal seed orchards*. - African Crop Science Journal, 15(2): 59-66.

NOWICKE J.W., SKVARLA J.J., 1981 - *Pollen morphology and phylogenetic relationships of the* Berberidaceae*.* -

Smithsonian Contributions to Botany, 50: 1-83.

O'BRIEN T.P., Mc CULLY M.E., 1981 - *The study of plant structure: principles and selected methods*. - Termarcarphy Pty, Melburne, Australia, pp. 357.

ONTIVERO M., RADICE S., GIORDANI E., BELLINI E., 2006 - *Effects of different pollination treatments in genotypes of* Prunus salicina *Lindl*. - International Journal of Horticultural Science, 12(2): 141-146.

ORSI M.C., 1984 - Berberidaceae, pp. 325-348. - In: CORREA M.N. (ed.) *Flora Patagónica Sección 4ª*. INTA, Buenos Aires, Argentina.

PAUPIÈRE M.J., VAN HEUSDEN A.W., BOVY A.G., 2014 - *The metabolic basis of pollen thermo-tolerance: perspectives for breeding*. - Metabolites, 4(4): 889-920.

SARI-GORLA M., FROVA C., 2005 - *Pollen tube growth and pollen selection*, pp. 333-351. - In: SHIVANA K.R., and V.K. SAWHNEY (eds.) *Pollen biotechnology for crop production and improvement*. Cambridge University Press, NY, USA, pp. 468.

TACÓN CLAVAÍN A., 2004 - *Manual de productos forestales no madereros.* - CIPMA, Valdivia, Chile, pp. 22.

TEJASWINI P., 2002 - *Variability of pollen grain features: a plant strategy to maximize reproductive fitness in two species of* Dianthus*?* - Sexual Plant Reproduction, 14: 347-353.

URQUIETA C.A.P., 2010 - *Evaluación de la viabilidad polínica de cuatro especies pertenecientes al género* Berberis *L. (*Berberidaceae*)*. - Thesis, Chile University, pp. 85.

VARIS S., REINIHARJU J., SANTANEN A., RANTA H., PULKKINEN P., 2011 - *The size and germinability of Scots pine pollen in different temperatures* in vitro*.* - Grana, 50(2): 129-135.

YISTRA B., TOURAEV A., MORENO R.M.B., STÖGER E., VAN TUNEN A.J., VICENTE O., HEBERLE-BORS E., 1992 - *Flavonols stimulate development, germination, and tube growth of tobacco pollen*. - Plant Physiology, 100(2): 902-907.

Adv. Hort. Sci., 2016 30(1): 39-45

Analysis of the effects of *Glomus etunicatum* fungi and *Pseudomonas fluorescence* bacteria symbiosis on some morphological and physiological characteristics of Mexican lime (*Citrus aurantifolia* L.) under drought stress conditions

A.R. Shahsavar [1 (*)], **A. Refahi** [1], **M. Zarei** [2], **E. Aslmoshtaghi** [1]

[1] *Department of Horticultural Science, College of Agriculture, Shiraz University, Shiraz, Iran.*

[2] *Department of Soil Science, College of Agriculture, Shiraz University, Shiraz, Iran.*

Key words: chlorophyll content, drought deficit, leaf water potential, Mexican lime.

Abstract: To analyze the effects of *Glomus etunicatum* fungi and *Pseudomonas fluorescence* bacteria on some morphological and physiological characteristics of Mexican lime plant under drought stress conditions, a factorial experiment was conducted. This experiment was based on a completely randomized design with three replicates; each replicate was composed of two pots. The factors used consisted of *G. etunicatum* fungi and control, *Pseudomonas fluorescence* bacteria and control, and drought stress at three levels (-0.35, -0.47, and -0.6 bars). The analyzed characteristics were leaf chlorophyll content, leaf temperature, rate of net photosynthesis, transpiration, leaf relative water content (RWC), and percentage of root colonization. Data analysis revealed that both fungi and bacteria increased leaf chlorophyll content, net photosynthesis rate, transpiration, and leaf RWC. Moreover, the presence of fungi reduced leaf temperature while inoculation of bacteria had no effects on that the parameter. In addition, with the increase of irrigation periods, leaf temperature and transpiration were also increased. Results showed that root colonization percentage dropped with increased irrigation and the highest root colonization percentage was observed in simultaneous inoculations of fungi and bacteria with a two-day irrigation period.

1. Introduction

Biological and non-biological stresses, which are mostly due to adverse weather conditions, are main factors in yield reduction (Wu *et al.,* 2006). There is much evidence that mycorrhizal fungi cause variations in plant-water relations and improve drought tolerance. Improvement in plant-water relations is affected by direct and indirect mechanisms (Davies *et al.,* 1993). In general, plants that have mycorrhizal symbiosis grow and perform better as they absorb more nutrients and water from the soil. These plants are also more tolerant towards environmental stresses including biotic and abiotic stresses (Porcel and Ruiz-Lozano, 2004). Most varieties of citrus, like orange, trifoliate orange, Cleopatra mandarins, Swingle citrumelo, and Citrange, are very dependent, because of their hairy roots, on *Glomus* species (Davies *et al.,* 1993). Plant adaptations to arid climate conditions, morphological and physiological changes, and concentration of novel metabolites along with structural variations, increase their efficiencies in stress conditions (Wu *et al.,* 2006).

When plants are under drought stress, osmotic adjustments occur to reduce potential water loss. This phenomenon leads to good water flow maintenance from the soil to plant roots (Porcel and Ruiz-Lozano, 2004). *G. versiforme* fungus increased leaf water potentials of trifoliate orange and mandarin seedlings under both drought stress and enough-water-supply conditions (Wu *et al.,* 2006, 2008). Moreover, when trifoliate orange seedlings were under drought stress, the leaf relative water content (RWC) significantly increased compared to plants with no fungus (Wu *et al.,* 2006). In mandarin seedlings, plant height, leaf area and number of leaves per plant, decreased under drought stress conditions, while all those factors were improved using *G. versiforme* fungi (Wu and Zou, 2009). In citrus plants, *G. versiforme* fungi increased growth and

(*) Corresponding author: shahsava@shirazu.ac.ir
Received for publication 31 October 2015
Accepted for publication 4 February 2016

biomass while they reduced their colonization percentage. These increases were attributed to the improvement of fungi water absorptions and increases in the length and volume of plant fungal roots (Faber *et al.,* 1991; Bryla and Duniway, 1997; Wu *et al*., 2011). In these plants, root colonization increases with a decrease in drought stress (Augé, 2001). Under drought stress conditions, *G. versiforme* fungi increased fresh and dry weight of plant roots and shoots and increased the root colonization percentage (Wu and Xia, 2006). *G. intraradices* fungus, under drought stress conditions, increased root growth and respiration rate of Rough lemon (Leyv and Syvestern, 2006). Many studies showed that *G. etunicatum* fungi could affect plant-water relations of host plants including citrus, under both drought stress and enough-water-supply conditions (Wu *et al.,* 2006). Therefore, they cause higher water use efficiency and this water use efficiency in mycorrhizal plants becomes even more tangible in drought stress conditions (Davies *et al.,* 1993). *Glomus etunicatum* fungus increased phosphorus, potassium, zinc, and copper in pistachio trees planted under sufficient water supply conditions and also increased nitrogen and calcium in pistachio trees planted under drought stress conditions. However, this fungus did not change the magnesium concentration (Abbaspour *et al.,* 2011). It is reported that *Pseudomonas* bacteria enhances growth and yield of some plants (Rodriguez and Fraga, 1999). Construction of active metabolites such as vitamins, amino acids, and Indole acetic bacteria may have a direct effect on the growth and metabolite contents of *Piriformospora indica* and mycorrhizal fungi. As a helpful microorganism, it seems that bacteria supports fungal performances (Vivas *et al.,* 2003). Plant inoculations with different types of *Pseudomonas* bacteria in drought stress situations increased plant proline contents, thus the plants' water levels were maintained and their protein contents and membranes remained safe from drought stress damage (Yoshiba *et al.,* 1997). Inoculation with *Pseudomonas* species, led to moderation of drought stress effects, improvement of plant growth and increase of proline, soluble sugars and amino acids production, explaining their effectiveness in absorbing water and nutrients from the soil (Wu *et al.,* 2008). These types of bacteria also help the plant maintain its RWC and LWL (leaf water loss) levels under drought stress conditions. Studies have shown that mycorrhizal plants absorb more CO_2 in the presence of light. Hence, their photosynthesis rates are also higher. The increase of CO_2 absorption in mycorrhizal plants is related to a decrease of liquid-phase resistance of mesophyll cells to CO_2 transmission (Wu and Zou, 2009). Miller (2000) reported that in mycorrhizal plants, due to the increase of photosynthesis materials and rate, water use efficiency increased per water use unit. Mycorrhiza can increase plant weight, leaf area, and plant pigments, and these increases may be attributed to the improvement of fungi water and phosphorus absorptions (Bethlenfalvay *et al.,* 1988; Davies *et al.,* 1993). *Glomus etunicatum* and *Pseudomonas* bacteria have positive effects on plant growth and employing them, instead of fertilizers, is considered a positive approach to reduce fertilizer use (Davies *et al.,* 1993). Despite the lack of comprehensive scientific investigations on the horticultural characteristics of Mexican lime (*Citrus aurantifolia* Swingle cv. Mexican Lime) as a rootstock, its seed availability for propagation and some its characteristics, such as good crop load and vigorous habit of grafted cultivars as scion, have made it a favorite in Fars province, Iran.

Considering the positive effects of fungi and bacteria in symbiosis with some plant roots, the aim of this study was to investigate the effects of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria and their interactions on some morphological and physiological characteristics of Mexican lime plant under drought stress conditions.

2. Materials and Methods

Preparation and inoculation of plant materials

Mexican lime seedlings, six months of age and disease-free, were provided in Khafr city of Fars province, Iran. They were transferred to the greenhouse. Planting soil mixture in ratio 1:1:1 (sand:soil: leaf compost) was sterilized and 2.7 kg were placed in plastic pots. The arbuscular mycorrhizal fungi isolate used in this study was *G. etunicatum* supplied from the soil lab of the Faculty of Shiraz University. The lyophilized fungal inoculum of *Pseudomonas fluorescence* was supplied from Tehran University School of Soil and Water and was prepared as follows. To prepare a solution containing growth-stimulating bacteria, a nutrient broth (NB) medium was applied. First, 0.8 g of NB was dissolved in 100 mg of distilled water and then media were sterilized by autoclaving at 121°C and pressure of 1.1 atm for 25

min. A lyophilised pre-culture vial was first suspended in 0.3 mL of nutritive medium. One drop (1 ml) of that suspension was added to 5 mL of nutritive medium and incubated on an orbital shaker at 28°C for 24 h. This final preparation of medium was used as the inoculum. After the incubation period, roots were placed in a solution containing bacteria for 30 min. Moreover, to ensure its effectiveness, 10 cc of the solution containing bacteria were added to each pot. For drought stress treatments, pots containing 2.7 kg soil without a seedling were selected and their moisture contents were equilibrated with the previously measured field capacity. The wet soils of the pots were weighed daily for 15 days, always at the time. Daily water reductions and moisture curves were graphed. Using those diagrams, irrigation periods were identified for every 2, 4 and, 6 days. For *G. etunicatum* fungus inoculation, 70 g of inoculum containing spores, hyphae, and root fragments were introduced 5 cm beneath the soil surface in the pots, and mixed thoroughly. Equal to the amount of added inoculum, hyphae, and mycelium to the fungal treatment pots, inoculum without hyphae and mycelium was added to control pots. For bacteria inoculation, seedlings were placed in a solution containing *Pseudomonas fluorescence* bacteria for 30 min and were then planted into pots. For fungi and bacteria treatments, bacteria-inoculated seedlings were planted in pots in which fungus was previously added. One seedling was planted per pot and, two months later, water treatments were applied. After six months, the implants were removed. The study was conducted using a factorial experiment, based on a completely randomized design with three replications in two replicate pots. Factors used in the experiment were: 1) *G. etunicatum* fungus in two levels of *G. etunicatum* and control; 2) Growth stimulating bacteria in two levels of *Pseudomonas fluorescence* and control; 3) Drought stress at three levels.

The Kormanik and McGraw method (Kormanik and McGraw, 1982) was used to measure colonization percentage. In this method, 2 g of roots previously stored in FAA (formaldehyde - acetic acid - ethanol) were washed with water three or four times and were placed in Falcon tubes containing 10% KOH solution for 24 h at room temperature. The color of the solution was almost yellow or light yellow. The solution was then poured out and the roots were again washed with water three or four times. The samples were placed in 2% hydrochloric acid for at least 15 min for staining. The acid was poured out and a colored solution was poured over the acidic roots. Acid fuchsine stain was used in this study; the ratio of the fuchsine acid colored solution was 14 ml lactic acid, 1 ml glycerin and, 1 ml water. The roots and the solution were kept at room temperature for 24 h. The coloring solution was then removed. Besides, due to elimination of extra colors, the coloring solution was poured on the roots. After 6-12 h, fungal organs such as arbuscules, hyphae, and vesicles were observed under a light microscope and colonization was calculated as a percentage. After application of water stress treatments, leaf chlorophyll content was measured with a SPAD-502 chlorophyllmeter using three fully-expanded leaves to find an average for chlorophyll content. Leaf temperature factors, net photosynthesis and transpiration rates were measured by portable photosynthesis meter (LCi, ADC, England). Relative water content was determined by using ten 7 mm-diameter leaf discs. Leaf discs for each treatment were weighed (FW). They were hydrated until saturation (constant weight) for 48 h at 5°C in darkness (TW). The leaf discs were then dried in an oven at 105°C for 24 h (DW). Relative water content was calculated according to the following expression (Filella *et al.,* 1998):

$$RWC\% = (FW\text{-}DW)/(TW–DW) \times 100$$

Statistical analysis

The data were analyzed for significance ($P<0.050$) by ANOVA (analysis of variance) with mean separation by Duncan's Multiple Range test.

3. Results and Discussion

Leaf relative water content (RWC)

Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria on Mexican lime leaf RWC, at different irrigation periods, identified that the maximum leaf RWC was observed in simultaneous inoculation of fungi and bacteria with the two-day irrigation period (74.7%). The general results indicate that the leaf RWC decreased with the increase in irrigation period, while inoculation with fungi or bacteria significantly increased RWC in all irrigation periods (Table 1).

Osmotic adjustment is one of the most important factors in plant drought tolerance and it is closely related to RWC (Haley *et al.,* 1993). When plants are under a drought stress condition, osmotic adjust-

ment occurs to reduce water potential and maintain a good flow of water from the soil to the plant roots. Plants with mycorrhizal fungi have more osmotic adjustment potentials than plants without fungi (Porcel and Ruiz-Lozano, 2004). Manette *et al*. (1988) reported that plants which are under drought stress conditions have specific morphological and physiological characteristics that enable them to store more water. Clarke and Craig (1982) stated that plants under drought stress conditions loose their water content more slowly. They also indicated that there are significant relationships between water content of the loss of leaves, plant drought tolerance, and leaves ability to retain water content (Clarke and Craig, 1982). Therefore, mycorrhizal plants have higher osmotic adjustment and are more capable of retaining their water content.

Table 1 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime leaf RWC with different irrigation periods (%)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	74.7 a	73.5 ab	72.9 ab	72.6 ab
4	71.9 b	70.3 b	69.5 bc	67.6 c
6	70.2 b	69.5 bc	68.4 bc	66.3 c

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

Chlorophyll content

Chlorophyll content decreased with the increase of irrigation periods. In addition, inoculations of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria increased leaf chlorophyll content. Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria leaf chlorophyll content identified that the maximum leaf chlorophyll content was observed when both fungi and bacteria were inoculated and there was a two-day irrigation period (634.7). The lowest chlorophyll content was observed in the treatment without fungi and bacteria inoculations with six-day irrigation periods (Table 2).

Analysis of the effects of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria inoculations on chlorophyll content of the Mexican lime leaves in the current study revealed that the chlorophyll content decreased with an increase of drought stress periods. However, inoculations of fungi and bacteria largely reduced the deleterious effects of drought. This can

Table 2 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime leaf chlorophyll content with different irrigation periods (SPAD value)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	634.7 a	574.3 b	565.4 b	529.6 c
4	578.2 b	512.9 cd	511.7 cd	441.7 f
6	503.8 d	484.6 e	479.3 e	320.5 g

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

be explained by the fact that in drought stress conditions, the chlorophyllase enzyme becomes activated while its activation results in the loss of chlorophyll content (Shaharoona *et al.*, 2008). Under drought, oxygen free radicals, which are damaging to various cellular organelles, are formed. One of the most sensitive organelles to drought stress and free radicals is chloroplast (Kaya *et al.*, 2003). *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria, by increasing antioxidant content and antioxidant enzyme activities, cause a loss of detrimental free radicals and consequently preserve plant chlorophyll content (Molinari *et al.,* 2007). They also increase the absorption of elements such as magnesium, iron, and nitrogen that lead to the plant's production of more chlorophyll (Molinari *et al.,* 2007).

Leaf temperature

Our results indicate that the increase of irrigation periods led to an increase of leaf temperature. The presence of *G. etunicatum* fungi decreased leaf temperature while *Pseudomonas fluorescence* bacteria inoculation had no effect on it. Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria on leaf temperature revealed that the minimum leaf temperature was with simultaneous inoculation of fungi without bacteria and a two-day irrigation period (31.47°C). Likewise, the maximum temperature was observed in the treatment without fungi and bacteria inoculations and a six-day irrigation periods (Table 3).

Rate of net photosynthesis

Analysis of the net photosynthesis rate of Mexican lime revealed that it declined with the increase of irrigation periods: the maximum and minimum rates were observed with two- and six-day irri-

Table 3 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime leaf temperature with different irrigation periods (°C)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	32.59 de	31.47 e	33.16 d	33.05 d
4	34.25 d	33.87 d	34.92 c	36.50 b
6	35.94 b	36.35 b	36.28 b	38.41 a

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

gation periods, respectively. The results also indicated that the presence of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria increased the plants' rate of net photosynthesis. Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria identified that the maximum rate was observed in simultaneous inoculation of both fungi and bacteria and with a two-day irrigation period (12.3 micromole/m^2/s) (Table 4).

Table 4 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime photosynthesis rate with different irrigation periods (micromole/m^2/s)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	12.3 a	11.6 ab	11.4 b	10.50 c
4	10.2 c	10.2 c	10.1 c	9.06 d
6	9.51 d	8.52 de	8.37 e	6.48 f

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

Rate of transpiration

Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria on Mexican lime transpiration rate in plants grown with different irrigation periods identified that the highest rate was observed in simultaneous inoculation of both fungi and bacteria and a two-day irrigation period (10.25 micromole/m^2/s). Likewise, the minimum transpiration rate was observed in the treatment without fungi and bacteria inoculations and a six-day irrigation period. The overall results showed that the leaf transpiration rate increased with the increase of irrigation period (Table 5).

Table 5 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime transpiration rate with different irrigation periods (micromole/m^2/s)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	10.25 a	9.96 b	10.07 ab	9.83 b
4	9.68 c	9.58 c	9.16 d	8.74 e
6	9.17 d	9.72 b	8.91 e	8.65 e

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

Wu and Xia (2006) specified that under drought stress conditions, *G. versiforme* fungi increase leaf water potential, photosynthesis rate, respiration rate, RWC, and stomatal conductance of mandarin seedlings; however, leaf temperature is decreased compared to plants without fungi. Effects of irrigation period on leaf temperature, photosynthesis rate, and transpiration showed that with the increase of irrigation period, they all declined (Figueiredo, 2008). This can be explained by the fact that under drought condition, more stomata are closed; with a loss of evaporation, the leaf surface loses less heat and the leaf temperature increases (Dietz and Foyer, 1986.). Moreover, because of stomata closure, less water is lost and the transpiration rate decreases. It should be noted that stomata closure causes less carbon dioxide to enter into the leaf, resulting in a lower rate of photosynthesis (Zhang *et al.,* 2010). The presence of *G. etunicatum* fungi and inoculation with *Pseudomonas fluorescence* bacteria leads to better water absorption and higher drought stress tolerance, thus increasing the plant's rate of photosynthesis. Many studies have reported the effects of *G. etunicatum* fungi on increasing photosynthesis rate (Johnson *et al.,* 1986), increasing root hydraulic conductivity for water uptake (Graham and Syvertsen, 1984), and increasing transpiration rate (Leyv and Syvestern, 2006).

Root colonization percentage

Results of the present study showed that root colonization occurred in the presence of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria. Moreover, an increase of irrigation period led to a decrease of root colonization percentage. Analysis of the effects of interaction between inoculation of *G.*

etunicatum fungi and *Pseudomonas fluore*scence bacteria on Mexican lime percentage of root colonization revealed that the maximum percentage was observed in simultaneous inoculation of both fungi and bacteria with a two-day irrigation period (49.66%) (Table 6).

Table 6 - Effects of *G. etunicatum* fungus and *Pseudomonas florescence* bacteria inoculations on Mexican lime root colonization percentage with different irrigation periods (%)

Irrigation periods (day)	GE +		GE -	
	PF +	PF -	PF +	PF -
2	49.66 a	42.36 ab	0 d	0 d
4	38.73 b	36.87 bc	0 d	0 d
6	34.24 c	35.12 c	0 d	0 d

In each column, means followed by different letters differ significantly at P≤0.05 according to Duncan's multiple range test. GE + = *G. etunicatum* presence; GE - = *G. etunicatum* absence. PF + = *Pseudomonas florescence* presence; PF - = *Pseudomonas florescence* absence.

As previously mentioned, root colonization occurred only in the presence of *G. etunicatum* fungi and its percentage dropped with an increase in irrigation period. Until now, no specific reason has been proposed for the reduction of colonization in drought stress conditions. Probably water is one important element in fungi growth. The formation of secondary metabolites that prevent fungi growth in the plant roots is also a possible explanation. Wu *et al*. (2006) reported that, in the case of citrus roots, the highest colonization percentage of mycorrhizal fungi occurs when the roots are not under drought stress conditions, which is consistent with the present study results. Regarding other types of citrus, they found similar results in their subsequent studies (Wu *et al.,* 2006, 2008). In order to utilize root colonization of fungi and bacteria capacities in sustainable agriculture, there must be appropriate establishment of both fungi and bacteria on the plant roots. Accordingly, observation of Mexican lime root colonization percentage in the current investigation was a very important and valuable factor. In addition, specification of the appropriate colonization percentage for effective interaction between fungi and plant is an important issue.

4. Conclusions

The results of the current study and other research projects in this field have shown the practical and scientific advantages of *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria applications in arid or semi-arid areas. The synergistic effect, which was observed between *G. etunicatum* fungi and *Pseudomonas fluorescence* bacteria, could increase most of the plant characteristics such as leaf chlorophyll content, net photosynthesis and transpiration rates, leaf RWC and root colonization percentage which provide the material energy and information for plant growth, development and reproduction. *Pseudomonas fluorescence* bacteria could reduce the negative effects of drought stress less than *G. etunicatum* fungi. Using their hyphae and extra/intra root mycelia, *G. etunicatum* fungi expand root evacuation area for better uptakes of water and nutrients. Arbuscular mycorrhizal fungi can be integrated in soil management to achieve low-cost sustainable agricultural systems, offering a sustainable and environmentally safe treatment to improve drought tolerance. Consequently, using these fungi as well as *Pseudomonas fluorescence* bacteria can be very effective in achieving the goals of sustainable agriculture.

References

ABBASPOUR H., SAEIDSARAND S., AFSHAR H., 2011 - *Improving drought tolerance of* Pistacia vera *L. seedlings by arbuscular mycorrhiza under greenhouse conditions*. - J. Medicinal Plants Research, 5: 7065-7072.

AUGÉ R.M., 2001 - *Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis.* - Micorrhiza, 11: 3-42.

BETHLENFALVAY G.J., BROWN M.S., AMES R.N., THOMAS R., 1988 - *Effects of drought on host and endophyte development in mycorrhizal soybeans in relation to water use and phosphate uptake*. - Physiol. Plant., 72: 565-571.

BRYLA D.R., DUNIWAY J.M., 1997 - *Effects of mycorrhizal infection on drought tolerance and recovery in safflower and wheat*. - Plant and Soil., 197(1): 95-103.

CLARKE J.M., CRAIG T.N., 1982 - *Excised-leaf water retention capability as an indicator of drought resistance of* Triticum *genotypes*. - Can. J. Plant Sci., 62: 571-578.

DAVIES F.T., POTTER J.R., LINDERMAN R.G., 1993 - *Drought resistance of mycorrhizal pepper plants independent of leaf P concentration response in gas exchange and water relations.* - Physiologia Plantarum., 87: 45-53.

DIETZ K.J., FOYER C., 1986 - *The relationship between phosphate status and photosynthesis in leaves.* - Planta, 167(3): 376-381.

FABER B.A., ZASOSKE R.J., MUNNS D.N., SHACKEL K., 1991

- *A method for measuring hyphal nutrition and water uptake in mycorrhizal plants*. - Can. J. Bot., 69: 87-94.

FIGUEIREDO V.B., 2008 - *Alleviation of drought stress in the common bean (*Phaseolus vulgaris *L.) by co-inoculation with* Paenibacillus polymyxa *and* Rhizobium tropici. - Appl. Soil Ecol., 40: 182-188.

FILELLA I., LLUSIA J., PIN J.O., PEN J.U., 1998 - *Leaf gas exchange and fluorescence of* Phillyrea latifolia*,* Pistacia lentiscus *and* Quercus ilex *saplings in severe drought and high temperature conditions*. - Environ. Exp. Bot., 39: 213-220.

GRAHAM H., SYVERTSEN J.P., 1984 - *Influence of vesicular-arbuscular mycorrhiza on the hydraulic conductivity of roots of two citrus rootstocks*. - New Phytologist., 97: 277-284.

HALEY S.D., QUICK J.S., MORGAN J.A., 1993 - *Excised-leaf water status evaluation and associations in field-grown winter wheat*. - Can. J. Plant Sci., 73:55-63.

JOHNSON C.R., DUKE E.R., KOCH K.E., 1986 - *Accumulation of phosphorus, dry matter and betaine during NaCl stress of split-root citrus seedlings colonized with vesicular-arbuscular mycorrhizal fungi on zero, one or two halves*. - New Phytologist., 104: 583-590.

KAYA C., HIGGS D., KIRNAK H., TAS I., 2003 - *Mycorrhizal colonization improves fruit yield and water use efficiency in watermelon (*Citrullus lanatus *Thunb.) grown under well-watered and water-stressed conditions*. - Plant Soil., 253: 287-292.

KORMANIK P.P., MCGRAW A.C., 1982 - *Quantification of vesicular-arbuscular mycorrhizae in plant root*, pp. 37-45. - In: SCHENK N.C. (ed.) *Methods and principles of mycorrhizal research*. The American Phytopathological Society, St. Paul, MN, USA, pp. 244.

LEYV Y., SYVESTERN J.P., 2006 - *Effect of drought stress and vesicular arbuscular mycorrhiza on citrus transpiration and hydraulic conductivity of roots*. - J. Plant Physiol., 85: 25-31.

MANETTE A.S., RICHARD C.J., CARVER B.F., MORNHINWEG D.W., 1988 - *Water relations in winter wheat as drought resistance indicators*. - Crop Sci., 28: 526-531.

MILLER M.H., 2000 - *Arbuscular mycorrhizae and the phosphorus nutrition of maize: A review of Guelph studies*. - Can. J. Plant Sci., 80: 47-52.

MOLINARI H.B., MARUR C.J., DAROS E., MARILIA CAMPOS K.F., CARVALHO J.F., FILHO J.C., PEREIRA L.F., VIEIRA L.G., 2007 - *Evaluation of the stress inducible production of proline in transgenic sugarcane (*Saccharum *spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress*. - Physiol. Plant., 130: 218-229.

PORCEL R., RUIZ-LOZANO J.M., 2004 - *Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress*. - J. Exp. Bot., 55: 1743-1750.

RODRIGUEZ H., FRAGA R., 1999 - *Phosphate solubilizing bacteria and their role in plant growth promotion*. - Biotech. Adv., 17: 319-339.

SHAHAROONA B., NAVEED M., ARSHAD M., ZAHIR Z.A., 2008 - *Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum *L.)*. - Appl. Microbiol. Biotechnol., 79: 147-155.

VIVAS A., JUAN B., RUIZ-LOZANO M., 2003 - *Influence of a* Bacillus *sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress.* - Mycorrhiza, 13: 249-256.

WU Q.S., XIA R.X., 2006 - *Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions*. - J. Plant Physiol., 163: 417-425.

WU Q.S., XIA R.X., ZOU Y.N., 2006 - *Reactive oxygen metabolism in nonmycorrhizal citrus (*Poncirus trifoliata*) seedlings subjected to water stress*. - J. Plant Physiol., 163: 1101-1110.

WU Q.S., XIA R.X., ZOU Y.N., 2008 - *Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress*. - Euro. J. Soil Biol., 44(1): 122-128.

WU Q.S., ZOU Y.N., 2009 - *The effect of dual application of arbuscular mycorrhizal fungi and polyamin upon growth and nutrient uptake of* Trifolia orange *seedling.* - Not. Bot. Agrobot. Cluj., 37(2): 95-98.

WU Q.S., ZOU Y.N., HE X., LUO P., 2011 - *Arbuscular mycorrhizal fungi can alter some root characters and physiological status in trifoliate orange (*Poncirus trifoliata *L. Raf.) seedlings.* - Plant Growth Regul., 65: 273-278

YOSHIBA Y., KIYOSUE T., NAKASHIMA K., YAMAGUCHI-SHINOZAKI K., SHINOZAKI K., 1997 - *Regulation of levels of proline as an osmolyte in plants under water stress*. - Plant Cell Physiol., 38: 1095-1102.

ZHANG Y., ZHONG C.L., CHEN Y., CHEN Z., JIANG Q.B., WU C., PINYOPUSAREK K., 2010 - *Improving drought tolerance of* Causarina equisetifilia *seedlings by arbuscular mycorrhizal under glasshouse conditions*. - New For., 40(3): 261-271.

Adv. Hort. Sci., 2016 30(1): 47-52

Induced resistance in potato plants by a non-pathogenic *Pseudomonas putida* BTP1 against potato tuber moth (*Phthorimaea operculella* Zeller)

A. Adam (*), I. Idris, N. Khalil, K. Houssian
Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS), P.O. Box 6091, Damascus, Syria.

Key words: biocontrol, plant resistance, potato tuber moth, *P. putida* BTP1, rhizobacteria.

Abstract: *Pseudomonas putida* strain BTP1 is able to promote induced systemic resistance (ISR) in a wide spectrum of pathosystems. In this study, we investigated induced resistance in potato plants against potato tuber moth (*Phthorimaea operculella* Zeller) by non-pathogenic *P. putida* BTP1. Several physiological indicators in the life cycle of the potato tuber moth, such as survival rate, mean weight of pupae, and sex ratio were studied to assess the protective effect of *P. putida* BTP1. Our results showed that treatment of potato tubers by bacterial suspension of *P. putida* BTP1 caused evident disturbance to the development of *P. operculella* in potato plants. Survival rate of larvae feeding on treated plant leaves and mean weight of pupae decreased significantly. In addition, a clear deviation in the sex ratio in moths, in favor of males, resulted from larvae fed on bacteria-treated plants. This study preliminarily reports the ability of BTP1 to induce resistance in potato plants against potato tuber moth. Consequently, *P. putida* strain BTP1 could be a promising approach for potato tuber moth biocontrol.

1. Introduction

The potato (*Solanum tuberosum* L.) has been considered one of the most important food crops, along with rice, wheat, and maize (Ross, 1986; Douches *et al.,* 2004). Potatoes grow in a variety of geo-environmental conditions. Developing countries cultivate potato to add nutritional balance to their food basket (Douches *et al.,* 2004; Navarre *et al.,* 2009). In Syria, more than 29,000 ha were planted with potato, producing about 609,000 t of tubers in 2005 (Alammouri, 2008). However, severe damage may occur to potato crops at storage periods particularly in developing countries. Within the Lepidoptera order, potato tuber moth *Phthorimaea operculella* (Zeller) belongs to the Gelechiidae family and it has been reported in more than 90 countries, making it a cosmopolitan pest (Visser, 2005; Golizadeh and Esmaeili, 2012). It damages potato throughout the growing season by mining stems, petioles, leaves and tubers by larvae, with the latter considered the typical damage. The procedure of potato damage begins when larvae penetrate the foliage, including leaves and stems. This insect can infest potato tubers stored and in field or it may develop on plants remaining in the field including tomatoes, aubergine or other solanaceous plants (Gilboa and Podoler, 1995; Coll *et al.,* 2000; Alvarez *et al.,* 2005). Farmers depend broadly on the use of insecticides and other varieties of farming practices (Clough *et al.,* 2008); insecticides are widely used to control this pest. However, insecticides are costly, nonselective, unfriendly to the environment, and affective for only a short period of time (Simmons *et al.,* 2006). Additionally, the phenomenon of resistance to insecticides in Lepidoptera has increased significantly (Gonzalez and Trevathan, 2001). Plant resistance, together with appropriate biological and farming practices in combination with insecticides may provide the best management options (Rondon, 2010).

Plants defeat pathogens through their active defense mechanisms that can be stimulated in some cases by plant growth-promoting rhizobacteria (PGPR) which ultimately reduce disease and render the host plant more resistant to any foreseeable pathogen attacks (Pieterse *et al.,* 2002). Induction of such enhanced defensive capacity is systemic as root

(*) Corresponding author: ascientific@aec.org.sy
Received for publication 9 August 2015
Accepted for publication 8 February 2016

treatment with a PGPR was shown to trigger protective effects on above-ground plant parts. These reactions are thought to typically result from the activation of latent defense mechanisms that are over-expressed upon subsequent pathogen challenge (Van Loon *et al.,* 1998; Ongena *et al.,* 2002; Bakker *et al.,* 2007). This induced systemic resistance (ISR) can be the basis for integrated plant disease management strategies (Ramamoorthy *et al.,* 2001; Zehnder *et al.,* 2001; Saravanakumar *et al.,* 2007). Induced resistance in plants by non-pathogenic rhizobacteria against pests is a very important additional factor for the protection of agricultural crops. Several studies have indicated the ability of many strains of rhizobacteria (PGPR) to induce systemic resistance against a large number of insect pests (Racke and Sikora, 1992; Zehnder *et al.,* 1997). For example, in cucumber against Striped Cucumber beetle *Acalymm avittatum* (Zehnder *et al.,* 1997, 2001), in cotton against American boll worm *Helicoverpa armigera* (Vijayasamundeeswari *et al.,* 2009), in tomato against whitefly *Bemisia tabaci* (Valenzuela-Soto *et al.,* 2010) and also in cucumber against spider mites *Tetranychus urticae* (Tomczyk, 2006). A non-pathogenic BTP1 showed enhancement of resistance level in many plants including bean, cucumber, and tomato against fungal pathogens (Ongena *et al.,* 2002, 2004; Adam *et al.,* 2008). In a previous study performed *in vitro* on grapevine rootstocks, we demonstrated the influence of *P. putida* BTP1 on reproduction and development of grapevine phylloxera (Adam *et al.,* 2013).

However, to our knowledge no studies have been performed yet to assess the effects of PGPR on *P. operculella* in potato plants. Therefore, the present work aims to demonstrate the protective effect triggered by *P. putida* strain BTP1 against *P. operculella* in potato plants. The larvae survival rate, the mean weight of pupae and the sex ratio were studied as a biometers to detect the induced resistance.

2. Materials and Methods

Establishment of the potato tuber moth colony

Insects used in the experiments were reared on waxed potato slices as described by Rahalakar *et al*. (1985). The experiments were conducted at a constant temperature of 25±1˚C with 70±5% RH, and a photoperiod of 12:12 (L:D) h.

Microbial strain and inoculum preparation

Psudomonas putida strain BTP1, isolated from barley roots, was originally selected for its specific features regarding pyoverdine-mediated iron transport (Jacques *et al.*, 1995; Ongena *et al.*, 2002). It was maintained and prepared for use in the ISR assays as previously described by Ongena *et al*. (2002). For the bioassays, BTP1 strain was grown in Erlenmeyer flasks (250 ml) containing 100 ml of Casamino Acids medium (CAA) for 24 h on a rotary shaker (150 rpm) at 28 oC. Cells were removed by centrifugation at 16500 g for 15 min at 4°C and washed in sterile NaCl (5g l^{-1}). The final pellet was resuspended in an adequate volume of sterile distilled water to obtain a bacterial suspension at 108 CFU ml^{-1}.

Assays for induced resistance

"Draja" potato tubers were washed in sterile water, dipped separately in a suspension of P. putida strain BTP1 for 30 min, and air-dried, while control tubers were treated with sterile water. The tubers were then planted in 10 L plastic pots containing autoclaved, moistened soil (three tubers/pot) to exclude any microorganism could affect BTP1. The pots were placed in a greenhouse at 25±1°C (day) and 23±1°C (night) with daylight of 16 h and relative humidity of 85-95%. Both control and treated plants were under the same watering and fertilizing conditions during the planting period.

Fresh leaves excised from potato plants (six to seven weeks old) were used for feeding the newly hatched larvae (24 h). For each treatment, 120 larvae in 10 (18 x 12 x 8 cm) plastic boxes (12 larvae/box) were fed on leaves until they reached the pupal stage. The boxes were resealed with parafilm to keep the larvae from escaping, and were then incubated at 25±1°C with daylight of 12 h and relative humidity of 70%. Each four-day-old pupae was weighed and placed separately within a small plastic tube. The pupae were classified into three groups according to their weights: small pupae (<5 mg), medium pupae (6-7 mg) and large pupae (>8 mg) to determine the larger sex. The number of pupae and the number of emerging moths (males or females) were recorded in order to calculate the survival rate of larvae and the sex ratio (the number of male/the number of female). The experiment was repeated three times.

Isolation of bacteria from potato plant leaves

Small leaf samples were taken from different parts of the potato plants treated with *P. putida*

BTP1. The samples were sterilized with sodium hypochlorite solution (5%) for 3 min and washed three times for 3 min. Samples were left to dry on sterile paper. They were then grown on Petri dishes containing the Casamino acid (CAA) medium. The dishes were incubated at 30±1°C for 72 h.

Statistical analysis

Statistical analyses were performed using STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level (P = 0.05). Data were subjected to analysis of variance (ANOVA) for the determination of differences between means. Differences between means of pupal weight were tested for significance using Tukey HSD test. Ratio Analysis test (Z-test) was used to compare the percentages of larval survival rate.

3. Results

Isolation of bacteria from potato plant leaves

The isolation of bacteria test on treated-potato plant leaves showed no bacterial colonies grew in the Petri dishes, indicating that *P. putida* BTP1 did not migrate through the plant (from the tubers to the leaves). There was no direct contact between bacteria and larvae.

Effect P. putida BTP1 on potato plants against potato tuber moth

Larvae survival rate. Induced resistance experiments showed the death of large numbers of larvae of *P. operculella* in different ages of development, particularly in *P. putida* BTP1-treated potato plants (Fig. 1.1). The larval survival rate decreased significantly (35%) when the larvae were fed on the excised leaves from *P. putida* BTP1-treated plants compared with the control plants (Fig. 1.2). Significant differences between BTP1 and control were observed in all experiments.

Effect on pupal weight. The results of three independent experiments showed that there was a negative impact on mean pupal weight in *P. putida* BTP1-treated potato plants. Where mean of pupal weight was 7.77±0.12 mg in the control potato plants, it decreased significantly in P. putida BTP1-treated plants to 6.24±0.15 mg (Fig. 2). This implies an approximately 20% weight reduction of pupae in potato plants pre-inoculated with *P. putida* BTP1 as compared with the control. Significant differences between BTP1 and control were observed in all experiments.

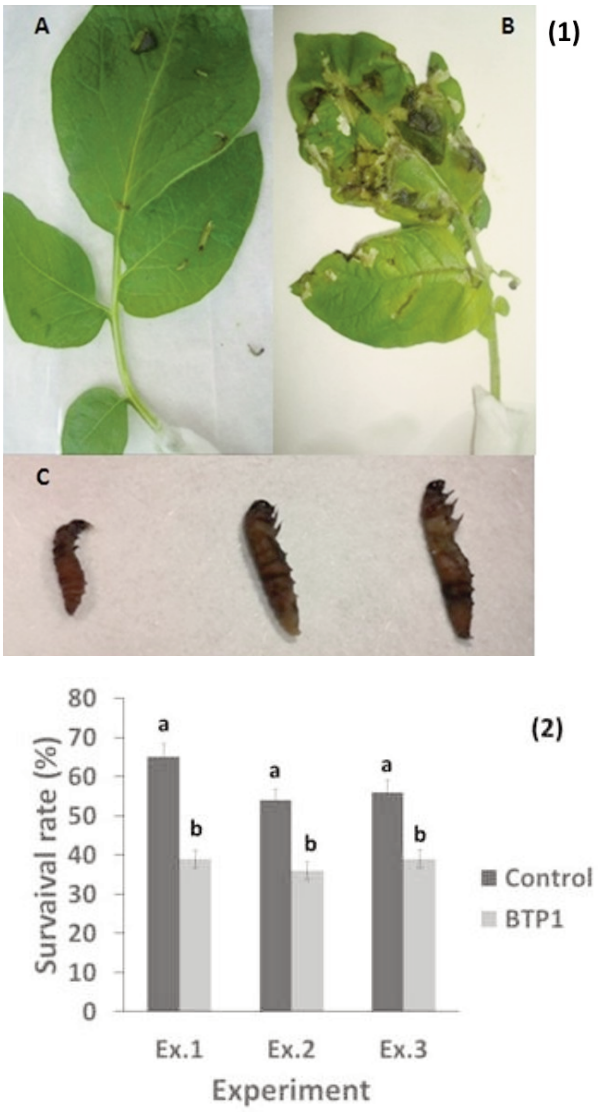

Fig. 1 - Example of potato leaves infested by potato tuber moth showing larvae feeding on *P. putida* BTP1-treated plant leaves (1A), and control plant leaves (1B). (1C): profile of the dead larvae in different stages because of malnutrition. (2): Influence of potato tuber treatment by the bacterial suspension of *P. putida* BTP1 on the survival rate of larvae of the potato tuber moth. Three separate experiments were carried out (120 larvae per treatment and per experiment were used). Data were subjected to ANOVA and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at P<0.001).

With regard to pupae weight, we observed that the pupae, which were classified into three groups according to their weight (small, <5 mg; medium, pupae 6-7 mg; large >8 mg), in control plants were mostly large pupae (approximately 61%) while the

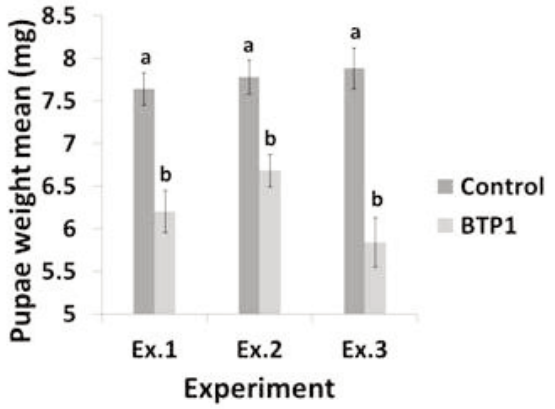

Fig. 2 - Influence of potato tuber treatment with bacterial suspension of *P. putida* BTP1 on mean of pupal weight of the potato tuber moth. Three separate experiments were carried out. Each column represents the weight mean of 25 pupae. Data were subjected to ANOVA and the differences between means were tested for significance using Tukey HSD test (values with different letters are significantly different at P<0.001).

small pupae was almost absent (3%) (Fig. 3). In contrast, in BTP1-treated plants, the percentage of the large pupae decreased significantly to reach (36%), while the percentage of the small pupae increased significantly to reach (27%) (Fig. 3).

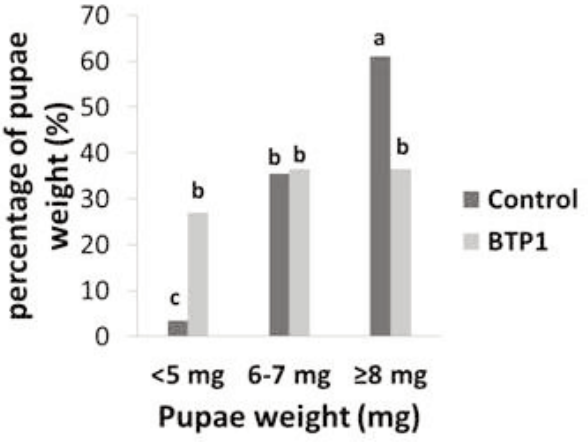

Fig. 3 - Influence of potato tuber treatment with bacterial suspension of P. putida BTP1 on percentages of pupal weight of the potato tuber moth (75 pupae/treatment). Data were subjected to test ratio analysis (Z-test) to determine the significant differences at P<0.05 (values with different letters are significantly different).

Sex ratio of P. operculella

The sex ratio of moths (number of males/number of females) emerged from control (180 moths) and *P. putida* BTP1-treated potato plants (91 moths) was calculated. A clear deviation was observed in the sex ratio in favor of males from feeding on BTP1-treated plants: 1.84:1, compared to 1:1 in control plants.

4. Discussion and Conclusions

Our study has shown that the larval survival rate and the mean of pupal weight were significantly decreased in BTP1-treated potato plants compared to control. These results are consistent with previous studies conducted on whitefly, which showed a significant decrease in survival rate (number of nymphs which are able to develop and reach the adult stage) in tomato plants treated by rhizobacteria (Valenzuela-Soto *et al.,* 2010). In addition, similar results were found in PGPR-treated cotton bolls with mortality of larval, malformation of pupal and adult with decreased adult emergence of American bollworm *H. armigera* (Vijayasamundeeswari *et al.,* 2009). Moreover, changes in dietary behavior of the rice leaf roller (*Cnaphalocrocis medinalis*) was observed, and there was a decrease in the larval and pupal weight in treated rice leaves by rhizobacteria (Radjacommare, 2002). The reduction of larval survival rate and pupal weight in BTP1-treated potato could be attributed to the inability of larvae to feed on treated plant leaves. It is well known that the growth of phytophagous larvae is affected indirectly by chemical or physical conditions or even both which characterize their host plants. For instance, PGPR-treated plants may have a decrease in essential nutrients or have compounds that inhibit growth, or both (Reese and Field, 1986; Bong and Sikorowski, 1991; Yaman *et al.,* 1999).

On the other hand, treatment of potato tubers with *P. putida* BTP1 also caused a clear deviation in sex ratio in favor of males. Quezada-Garcia *et al.* (2014) proved that nutritional variation causes differential mortality to the larger sex and the most sensitive to nutritional stress (female) in spruce budworm (*Choristo neural fumiferana* (Clemens); Lepidoptera). In contrast, House *et al*. (2011) demonstrated that offspring mortality in the dung beetle (*Onthopha gustaurus*; Coleoptera) vitally depends on the amount of resources that females have provisionally. In addition, they also showed that males have greater nutritional demands than females during development, which ultimately leads to higher mortality in the male population (the larger sex and the

most sensitive to nutritional stress) (House *et al.,* 2011; Quezada-Garcia *et al.,* 2014). These findings are consistent with our results which showed that females were larger (16.74%) than males.

In conclusion, understanding the mechanisms of induced defense by *P. putida* BTP1 is very important to enhance the resistance in potato plants. The current study provides evidence that *P. putida* strain BTP1 has a protective effect in potato plants against potato tuber moth. Based on similar studies that illustrated that the accumulation of some toxic phenolic compounds in the cells of resistant plants led to an increase in the death rate in insects, we believe that the treatment of potato tubers with *P. Putida* BTP1 leads to secondary metabolic changes in treated plant cells which elicit the production of defense compounds (Lattanzio *et al.,* 2000; Zehnder *et al.,* 2001; Arimura *et al.,* 2005; Melvin and Muthukumaran, 2008).

This study preliminarily reports the ability of *P. putida* strain BTP1 to induce resistance in potato plants against potato tuber moth. This bacterial strain could be a promising agent for potato tuber moth biocontrol. However, the controlled environment (plastic pots, sterile soil, and humidity) may lead to different results compared to farm applications due to competitors, T/HR condition, dispersion of inoculum, etc. Consequently, more research is needed to determine the mechanisms of defense induced in potato plants.

Acknowledgements

The authors thank Prof. I. Othman (Atomic Energy Commission of Syria) and Dr. N. Mirali (Department of Biotechnology) for their help. We thank Prof. P. Thonart and Dr. M. Ongena of the University of Liege, who provided us with the *P. putida* BTP1 strain.

References

ADAM A., IDRIS I., AYYOUBI Z., 2013 - In vitro Pseudomonas putida *BTP1-induced systemic resistance in grapevine rootstocks against phylloxera (*Daktulosphaira vitifoliae*)*. - Adv. Hort. Sci., 27(4): 137-142.

ADAM A., ONGENA M., DUBY F., DOMMES J., THONART P., 2008 - *Systemic resistance and lipoxygenase-related defence response induced in tomato by* Pseudomonas putida *strain BTP1*. - BMC Plant Biology, 8: 113.

ALAMMOURI N., 2008 - *Agricultural commodity outlook of Syrian potato*. - Ministry of Agriculture and Agrarian Reform, NAPC National agricultural policy center, Syria, pp. 23.

ALVAREZ J.M., DOTSETH E., NOLTE P., 2005 - *Potato tuberworm a threat for Idaho potatoes*. - University of Idaho, College of Agricultural and Life Sciences, Moscow, ID, USA, CIS 1125: 1-4.

ARIMURA G.-I., KOST C., BOLAND W., 2005 - *Herbivore-induced, indirect plant defences*. - Biochim. Biophys. Acta, 1734(2): 91-111.

BAKKER P.A.H.M., PIETERSE C.M.J., VAN LOON L.C., 2007 - *Induced Systemic resistance by fluorescent Pseudomonas spp.* - Phytopathology, 97(2): 239-243.

BONG C.F.J., SIKOROWSKI P.P., 1991 - *Effects of cytoplasmic polyhedrosis virus and bacterial contamination on growth and development of corn earworm, Heliothis zeae (Boddie)*. - Journal of Invertebrate Pathology, 57: 406-412.

CLOUGH G., DEBANO S., RONDON S., DAVID N., HAMM P., 2008 - *Use of cultural and chemical practices to reduce tuber damage from the potato tuberworm in the Columbia Basin*. - HortScience, 43: 1159-1160.

COLL M., GAVISH S., DORI I., 2000 - *Population biology of the potato tuber moth,* Phthorimaea operculella *(Lepidoptera: Gelechiidae) in two potato cropping systems in Israel*. - Bul. Entomol. Res., 90: 309-315.

DOUCHES D.S., PETT W., SANTOS F., COOMBS J., GRAFIUS E., LI W., METRY E.A.*,* NASR EL-DIN T., MADKOUR M., 2004 - *Field and storage testing* Bt *potatoes for resistance to potato tuberworm (Lepidoptera: Gelechiidae)*. - J. Econ. Entomol., 97: 1425-1431.

GILBOA S., PODOLER H., 1995 - *Presence-absence sequentials sampling for potato tuberworm (Lepidoptera: Gelechiidae) on processing tomatoes: selection of sample sites according to predictable seasonal trends*. - J. Econ. Entomol., 88: 1332-1336.

GOLIZADEH A., ESMAEILI N., 2012 - *Comparative life history and fecundity of* Phthorimaea operculella *(Lepidoptera: Gelechiidae) on leaves and tubers of different potato cultivars*. - J. Econ. Entomol., 105: 1809-1815.

GONZALEZ M.S., TREVATHAN L.E., 2001 - *Effect of biological and chemical seed treatment on seedling disease of soft red winter wheat in Mississippi*. - Seed Sci. Technol., 29(2): 413-427.

HOUSE C.M., SIMMONS L.W., KOTIAHO J.S., TOMKINS J.L., HUNT J., 2011 - *Sex ratio bias in the dung beetle Onthophagus taurus: adaptive allocation or sex-specific offspring mortality?* - Evol. Ecol., 25: 363-372.

JACQUES P., ONGENA M., GWOSE I., SEINSCHE D., SCHRODER H., DELFOSSE P.*,* THONART P., TARAZ K., BUDZIKIEWICZ H., 1995 - *Structure and characterization of isopyoverdin from* Pseudomonas putida *BTP1 and its relation to the biogenetic pathway leading to pyoverdines*. - Z. Naturforsch, 50: 622-629.

LATTANZIO V., ARPAIA S., CARDINALI A., VENERE D.D., LINSALATA V., 2000 - *Role of endogenous flavonoids in resistance mechanism of vigna to Aphids*. - J. Agric. Food Chem., 48(11): 5316-5320.

MELVIN J.M., MUTHUKUMARAN N., 2008 - *Role of Certain Elicitors on the Chemical Induction of Resistance in Tomato against the Leaf Caterpillar Spodoptera litura Fab.* - Not. Bot. Hort. Agrobot. Cluj, 36(2): 71-75.

NAVARRE D.A., GOYER A., SHAKYA R., 2009 - *Nutritional value of potatoes: vitamin, phytonutrient and mineral content*, pp. 395-324. - In: SINGH J., and L. KAUR (eds.) *Advances in potato chemistry and technology*. Elsevier Inc., Amsterdam, The Netherlands, pp. 508.

ONGENA M., DUBY F., ROSSIGNOL F., FAUCONNIER M.L., DOMMES J., THONART P., 2004 - *Stimulation of the lipoxygenase pathway is associated with systemic resistance induced in bean by a nonpathogenic* Pseudomonas *strain*. - Mol. Plant Microbe Interact., 17(9): 1009-1018.

ONGENA M., GIGER A., JACQUES P., DOMMES J., THONART P., 2002 - *Study of bacterial determinants involved in the induction of systemic resistance in bean by* Pseudomonas putida *BTP1*. - Eur. J. Plant Pathol., 108(3): 187-196.

PIETERSE C.M.J., VAN WEES S.C.M., TON J., VAN PELT J.A., VAN LOON L.C., 2002 - *Signaling in rhizobacteria-induced systemic resistance in* Arabidopsis thaliana. - Plant Biology, 4(5): 535-544.

QUEZADA-GARCIA R., PURESWARAN D., BAUCE E., 2014 - *Nutritional stress causes male-biased sex ratios in eastern spruce budworm (Lepidoptera: Tortricidae)*. - Can. Entomol., 146: 219-223.

RACKE J., SIKORA R.A., 1992 - *Isolation, formulation and antagonistic activity of rhizobacteria towards the potato cyst nematode,* Globodera pallida. - Soil Biol. Biochem., 24: 521-526.

RADJACOMMARE R., NANDAKUMAR R., KANDAN A., SURESH S., BHARATHI M., RAGUCHANDER T., SAMIYAPPAN R., 2002 - Pseudomonas fluorescens *based bioformulation for the management of sheath blight and leaffolder in rice*. - Crop Prot., 21: 671-677.

RAHALAKAR G.W., HARWALKAR M.R., RANANAVARE H.D., TAMHANKAR A.J., SHANTHRAM K., 1985 - Rhynchophorus ferrugineus, pp. 279-286. - In: SINGH P., and R.F. MOORE (eds.) *Handbook of insect rearing*. Elsevier, New York, USA, pp. 514.

RAMAMOORTHY V., VISWANATHAN R., RAGUCHANDER T., PRAKASAM V., SAMIYAPPAN R., 2001 - *Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases*. - Crop Prot., 20(1): 1-11.

REESE J.C., FIELD M.D., 1986 - *Defense against insect attack in susceptible plants: Black cutworm (Lepidoptera: Noctuidae) growth on corn seedlings and artificial diet*. - Ann. Entomol. Soc. Am., 79(2): 372-376.

RONDON S.I., 2010 - *The potato tuberworm: A literature review of Its biology, ecology, and control.* - Am. J. Potato Res., 87: 149-166.

ROSS H., 1986 - *Potato breeding. Problems and perspectives*. - Verlag Paul Parey, Berlin, Germany, 123.

SARAVANAKUMAR D., MUTHUMEENA K., LAVANYA N., SURESH S., RAJENDRAN L., RAGUCHANDER T., SAMIYAPPAN R., 2007 - *Pseudomonas-induced defence molecules in rice plants against leaffolder (*Cnaphalocrocis medinalis*) pest*. - Pest Manag. Sci., 63(7): 714-721.

SIMMONS A.T., NICOL H.I., GURR G.M., 2006 - *Resistance of wild lycopersion species to the potato moth* Phthorimaea operculella *(Zeller) (Lepidoptera: Gelechiidae)*. - Aust. J. Entomol., 45(1): 81-86.

TOMCZYK A., 2006 - *Increasing cucumber resistance to spider mites by biotic plant resistance inducers*. - Biological Lett., 43(2): 381-387.

VALENZUELA-SOTO J.H., ESTRADA-HERNANDEZ M.G., IBARRA-LACLETTE E., DELANO-FRIER J.P., 2010 - *Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting* Bacillus subtilis *retards whitefly* Bemisia tabaci *development*. - Planta, 231: 397-410.

VAN LOON L., BAKKER P., PIETERSE C., 1998 - *Systemic resistance induced by rhizosphere bacteria*. - Annu. Rev. Phytopatol., 36: 453-483.

VIJAYASAMUNDEESWARI A., LADHALAKSHMI D., SANKARALINGAM A., SAMIYAPPAN R., 2009 - *Plant growth promoting rhizobacteria of cotton affecting the developmental stages of Helicoverpa armigera*. - J. Plant Res., 49(3): 239-243.

VISSER D., 2005 - *Guide to potato pests and their natural enemies in South Africa.* - Arc- Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria, South Africa.

YAMAN M., DEMIRBAG Z., BELDUZ A.O., 1999 - *Investigations on the bacterial flora as a potential biocontol agent of chestnut weevil,* Curculio elephas *(Coleoptera: Curculionidae) in Turkey*. - African Journal of Mycology Biotechnology, 54: 625-630.

ZEHNDER G., KLOEPPER J., YAO C., WEI G., 1997 - *Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth-promoting rhizobacteria*. - J. Econ. Entomol., 90(2): 391-396.

ZEHNDER G.W., MURPHY J.F., SIKORA E.J., KLOEPPER J.W., 2001 - *Application of rhizobacteria for induced resistance*. - Eur. J. Plant Pathol., 107(1): 39-50.

Adv. Hort. Sci., 2016 30(1): 53-56

Occurrence of viruses in Calla and Peruvian lily in Tuscan nurseries and evidence of new viral records in Italy

A. Luvisi [1,2 (*)], D. Rizzo [3], L. Stefani [3], A. Panattoni [2], A. Materazzi [2]

[1] *Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Centro Ecotekne Università del Salento, Via Provinciale Lecce Monteroni, 73100 Lecce, Italy.*

[2] *Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, Via del Borghetto, 80, 56124 Pisa, Italy.*

[3] *Servizio Fitosanitario Regionale, Regione Toscana, Via Ciliegiole, 99, 51100 Pistoia, Italy.*

Key words: Alstroemeria, RT-PCR, *Zantedeschia*.

Abstract: In order to evaluate the health status of Calla and Peruvian lily in Tuscan nurseries, 18 viruses belonging to six families and one unassigned virus were assayed. Tests were carried out on 90 *Zantedeschia aethiopica* plants and 48 *Alstroemeria* spp. plants collected from 12 Tuscan nurseries in two years, via RT-PCR tests. *Z. aethiopica* was mainly affected by viruses belonging to the *Potyviridae* family, with the main infection caused by Dasheen mosaic virus (DsMV) and Zantedeschia mild mosaic virus (ZaMMV). Even if *Alstroemeria* spp. plants were affected by *Potyviridae* family viruses too, higher infection rates were recorded for *Betaflexiviridae*, where Lily symptomless virus infected more than half of plants. This is the first known report of Lily mottle virus (LMoV) in *Alstroemeria* spp. and *Z. aethiopica* or ZaMMV in *Alstroemeria* spp. in Italy.

1. Introduction

Calla and Peruvian lily are commonly cultivated in Tuscany (Italy) and they represent one of the main cut flower productions. Calla lily [*Zantedeschia aethiopica* (L.) Spreng] is known to be susceptible to at least 13 virus species, mainly belonging to *Potyviridae*, *Bunyaviridae,* and *Tombusviridae* (Huang *et al*., 2007). Peruvian lily (*Alstroemeria* spp.) has become one of the most popular cut flowers worldwide. It has been reported as the natural host of various plant viruses, including members of *Potyviridae*, *Betaflexiviridae* or *Bunyaviridae* (Park *et al*., 2010).

In Tuscany, virus surveys were carried out on various woody plants such as grapevine (Rizzo *et al*., 2012; 2015 a) but to our knowledge no reports are available for Calla and Peruvian lily in Italy. In order to evaluate the health status of these plants in Tuscan nurseries, various viruses were assayed, belonging to the following families: *Betaflexiviridae* [Alstroemeria carla virus (AlCV), Lily symptomless virus (LSV)], *Bromoviridae* [Cucumber mosaic virus (CMV)], *Bunyaviridae* [Impatiens necrotic spot virus (INSV), Iris yellow spot virus (IYSV), Tomato spotted wilt virus (TSWV)], *Comoviridae* [Arabis mosaic virus (ArMV), Broad bean wilt virus 1 (BBWV-1), Broad bean wilt virus 2 (BBWV-2)], *Potyviridae* [Alstroemeria mosaic virus (AlMV), Bean yellow mosaic virus (BYMV), Dasheen mosaic virus (DsMV), Konjac mosaic virus (KoMV), Lily mottle virus (LMoV), Turnip mosaic virus (TuMV), Zantedeschia mosaic virus (ZaMV), Zantedeschia mild mosaic virus (ZaMMV)], *Tombusviridae* [Carnation mottle virus (CarnMV)] and unassigned [Tobacco rattle virus (TRV)]. An additional aim of this survey was to evidence new viral records in Italy for these widespread cultivations, due to the intense international exchanges that characterize the commercialization of lily.

2. Materials and Methods

Tests were carried out on 90 *Z. aethiopica* plants and 48 *Alstroemeria* spp. plants collected from 12 Tuscan nurseries in two years. Plants showed symp-

(*) Corresponding author: andrea.luvisi@unisalento.it
Received for publication 24 November 2015
Accepted for publication 22 February 2016

toms such as foliar chlorosis, yellow spot and stripes. Total RNA was extracted from foliar tissue (2 g) using RNeasy Plant Mini Kit (Qiagen, Netherlands) protocol, modified according to MacKenzie *et al*. (1997). Tissues (2 g) were ground using a Tissue lyser (Qiagen) adding 5 ml of grinding buffer (4.0 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% PVP-40 and 2.0% sodium bisulfate) just before use. The homogenate (1 ml) was transferred to a 1.5 ml tube and 100 µl of 20% sarkosyl were added. After 2 min centrifugation, 600 µl were transferred to a QIAshredder spin column (Qiagen) placed in a 2 ml collection tube. The subsequent steps of RNA extraction were according to the manufacturer's protocol. The extracted RNA was then retro-transcribed into cDNA using the iScript cDNA Synthesis kit (Biorad, USA). For each sample, 2 µl of cDNA were amplified in a total volume of 20 µl containing 1X HotMaster Buffer, 0.5 µg/µl BSA and 1 U of HotMaster Taq DNA Polymerase (Eppendorf, Germany). Primers and RT-PCR parameters were chosen following the protocols reported in Table 1. 18S rRNA was used as internal control (Osman and Rowhani, 2006). Finally, 10 µl of the amplification mix was electrophoresed in a 1.5% agarose gel in TAE buffer [40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA pH (8.0)]. The amplified cDNA fragments were visualized on a UV transilluminator.

Data were analyzed using Sigma-Plot software (version 11; Systat Software, San Jose, CA). The software was used to perform analysis of variance (ANOVA). Data expressed in percent were converted to arcsin values. $P < 0.05$ was considered to be significant.

Table 1 - List of references for primers and RT-PCR conditions

Target	RT-PCR assay
Comoviridae	
ArMV	Faggioli *et al*., 2005
BBWV-1	Ferrer *et al*., 2008
BBWV-2	Ferrer *et al*., 2008
Betaflexiviridae	
AlCV	Spence *et al*., 2000
LSV	Lim *et al*., 2009
Bromoviridae	
CMV	Faggioli *et al*., 2005
Bunyaviridae	
IYSV	Kritzman *et al*., 2000
INSV	Liu *et al*., 2009
TSWV	Mumford *et al*., 1994
Potyviridae	
AlMV	Spence *et al*., 2000
BYMV	Ganesh Selvaraj *et al*., 2009
DsMV	Wen-Chi *et al*., 2010
KoMV	Wen-Chi *et al*., 2010
LMoV	Lim *et al*., 2009
TuMV	Wen-Chi *et al*., 2010
ZaMV	Kwon *et al*., 2003
ZaMMV	Wen-Chi *et al*., 2010
Tombusviridae	
CarnMV	Cevik *et al*., 2010
Unassigned	
TRV	Wei *et al*., 2009

3. Results

The health status of Calla and Peruvian lily as determined by the present survey is reported in Table 2. *Z. aethiopica* was mainly affected by viruses belonging to the *Potyviridae* family. More than two plants out of three were infected by DsMV and 50%

Table 2 - Health status of Calla lily (*Zantedeschia aethiopica*) and Peruvian lily (*Alstroemeria* spp.) expressed as percentage of infected plants

Target	*Zantedeschia aethiopica*	*Alstroemeria* spp.
Comoviridae		
ArMV	-	-
BBWV-1	-	-
BBWV-2	-	-
Betaflexiviridae		
AlCV	-	-
LSV	-	56.3 a
Bromoviridae		
CMV	-	12.5 b
Bunyaviridae		
IYSV	-	-
INSV	3.3 c *	-
TSWV	3.3 c	-
Potyviridae		
AlMV	-	-
DsMV	66.7 a	13.5 b
KoMV	-	-
LMoV	16.7 b	12.5 b
TuMV	-	-
ZaMMV	50.0 a	6.3 c
Tombusviridae		
CarnMV	-	-
Unassigned		
TRV	-	-

* Values in the same column followed by the same letter do not differ significantly according to Duncan's multiple range test (P=0.05).

Table 3 - Sequence of isolates of Zantedeschia mild mosaic virus and Lily mottle virus isolate detected in Italy

Zantedeschia mild mosaic virus isolate 2079 polyprotein gene, partial cds (GenBank: KF156666)
TCATTGAGTACCAACCCCAACAGTCCGATCTGTTTAATACTCGCGCGTCACAAACCCAATTCAATAATTGGTATGATGCGATCAAAAATGAG-TATGGGGTTGATGATAGTCAGATGCAGAGAATCATGAATGGCTTCATGGTGTGGTGTCTCGAGAATGGGACATCACCAAACATAAATGGCGTGTGGGT-TATGATGGATGGGGATGAACAAGTAGAATTTCCACTAAAACCAATGGTGGAGAATGCCAAGCCTACGCTGCGTCAAATAATGCACCACTTTTCAGACG-CAGCCGAGGCTTACATTGAACTTAGGAATGCCGCTGCCCCATATATGCCTAGATATGGGTTGCTGCGGAACTTAAGAGACAGAGGTCTAGCACGCTTCG-CATTCGACTTCTATGAAGTCACTTCAAAGACACCAGATCGTGCTAGAGAAGCTGTAGCGCAGATGAAGGCAGCAGCGCTAAACAATGTTTCCACAAG-GATGTTTGGATTGGATGGAAATATTGCAACTGCCACGGAGAACACTGAAAGGCACACTGCTAAGGATGTAAGTCCGAGCATGCACTCGCTACTCGG-GATCTCAGCCTTGCAGTAAAGGAGCTGGAAACAGCCCACAGTTATTGTCTTGCGATAGGGTTTAAATAGCCGTACTATTGTCGTTGCTAGATGTTG-CAGTGTGGGCCTCCCACCTTAAGGTTTATCAGTGTGGCTTTCCACCTAGTTCCTTACATTGCGCATAGTATGTGT
Lily mottle virus isolate 2409 coat protein gene, partial cds (GenBank: KF156662.1)
TGCTGGGGCCTCTAGCTCCACACAAACGAGTCGCCCAACACGTCCAGAGATTGCCGCGGTCGATGTAGCACCACAACAGAGCTCTGAGGCTA-GAGTGCGTGATCGTGATGTTGATGCTGGCACCGTGGGAACATACCAAATCCCTCGACTGAAAGCACTGGCAACAAAGATTAACGTACCCAAGGT-CAAGGGGCGAACAATAGTGAACACTGGGCACCTTGTGAACTACAACCCAGACCAAACAGATATTTCAAATACAAGGTCAACCCAGAAGCAATTTGA-GACCTGGCATAACGCTGTGAAAGATGAGTATGGTCTCAACGACGAGAGTATGGCTCTCGCAATGAATGGTCTGATGGTTTGGTGCATAGAGAATGG-CACCTCACCAAACATAAATGGCGTGTGGCTCATGATGGACGGAGATCAGCAAGTTGAATTTCCTTTACGTCCTATACTTGAACACGCAAAACC-GACGCTGCGCCAAATTATGGCGCATTTCTCAAACCTCGCTGAAGCTTATATTGAGAAGCAAAATTTGGAGAAACCGTACATGCCTAGGTACGGCCTT-CAGCGAAATCTCACCGATTTCAATCTAGCACGATTTGCTTTTGATTTCTATGA

of tested plants were infected by ZaMMV. Lower infection rates were reported for viruses belonging to *Bunyaviridae.* Even if *Alstroemeria* spp. plants were affected by *Potyviridae* viruses too, higher infection rates were recorded for *Betaflexiviridae*, where LSV infected more than half of plants. Further infections were caused by *Bromoviridae* viruses. *Comoviridae*, *Tombusviridae* or TRV infections were not found in both plants.

In *Z. aethiopica*, mixed infections were set at 40% for DsMV/ZaMMV, 6.7% for DsMV/LMoV/ZaMMV and 3.3% for DsMV/LMoV(data not shown). In *Alstroemeria* spp., mixed infections were set at 6.3% for LSV/ZaMMV, LSV/LMoV, DsMV/ZaMMV, CMV/LSV or CMV/DsMV/LSV.

With regard to *Alstroemeria* spp., the sequence obtained from a ZaMMV amplicon (GenBank accession no. KF156666 (Table 3) had 99% nucleotide identity with the corresponding fragment of a reference ZaMMV isolate (GenBank accession no. AY626825). Further isolates of LMoV were detected (GenBank accessions no. KF156667, KF156668, KF156669). Each further isolate had 99% nucleotide identity with the corresponding fragment of a reference ZaMMV isolate. All four isolates are different but had 99% nucleotide identity.

The sequence obtained from LMoV amplicon (GenBank accession no. KF156662) (Table 3) had 94% nucleotide identity with the corresponding fragment of a reference LMoV isolate (GenBank accession no. JN703466). The same LMoV amplicon was obtained from a *Z. aethiopica* sample as well. Further isolates of LMoV were detected in both species with 100% nucleotide identity with KF156662 (GenBank accessions no. KF156663, KF156664, KF156665).

4. Conclusions

Various viral infections, mainly due to viruses belonging to two families, *Betaflexiviridae* and *Potyviridae*, seem to affect Calla and Peruvian lily in Tuscan nurseries. Most detected viruses are frequently reported for both plants and mixed infection of virus belonging to *Potyviridae* are quite common in *Z. aethiopica* (Huang *et al*., 2007). However, some evidence is rarer.

To our knowledge this is the first report of LMoV in *Alstroemeria* spp. and *Z. aethiopica* or ZaMMV in *Alstroemeria* spp. in Italy, while ZaMMV was recently identified in Taiwan (Huang and Chang, 2005) and in Italy (Rizzo *et al*., 2015 b).

This report puts in evidence how widespread the virus is within these common plants and the need for constant monitoring of the health status for flower production. Even if some of viruses that affect Calla and Peruvian lily may be eradicated by thermotherapy (Panattoni *et al*., 2013) and heat treatment may help in *Bromoviridae* control (Luvisi *et al*., 2015), prevention represents the preferred method of virus control.

References

CEVIK B., BAKIR T., KOCA G., 2010 - *First report of Carnation mottle virus in Turkey*. - Plant Pathol., 59: 394.

FAGGIOLI F., FERRETTI L., ALBANESE G., SCIARRONI R., PASQUINI G., LUMIA V., BARBA M., 2005 - *Distribution of olive tree viruses in Italy as revealed by one-step RT-PCR*. - J. Plant Pathol., 87: 49-55.

FERRER R., ESCRIU F., LUIS-ARTEAGA M., GUERRI J., MORENO P., RUBIO L., 2008 - *New molecular methods for identification of Broad bean wilt virus 1*. - Mol. Cell. Probes, 22: 223-227.

GANESH SELVARAJ D., POKORNY R., HOLKOVA L., 2009 - *Comparative analysis of ELISA, one step RT-PCR and IC-RT-PCR for the detection of bean yellow Mosaic virus in gladiolus*. - Commun. Agric. Appl. Biol. Sci., 74: 853-859.

HUANG C.H., CHANG Y.C., 2005 - *Identification and molecular characterization of Zantedeschia mild mosaic virus, a new calla-lilly-infecting potyvirus*. - Arch. Virol., 150: 1221-1230.

HUANG C.H., HU W.C., YANG T.C., CHANG Y.C., 2007 - *Zantedeschia mild mosaic virus, a new widespread virus in calla lily, detected by ELISA, dot-blot hybridization and IC-RT-PCR*. - Plant Pathol., 56: 183-189.

KRITZMAN A., BECKELMAN H., ALEXANDROV S., COHEN J., LAMPEL M., ZEIDAN M., RACCAH B., GERA A., 2000 - *Lisianthus leaf necrosis: A new disease of Lisianthus caused by Iris yellow spot virus*. - Plan Dis., 84: 1185-1189.

KWON S.B., HA J.H., YOON J.Y., RYU K.H., 2003 - *Zantedeschia mosaic virus causing leaf mosaic symptom in calla lily is a new potyvirus*. - Arch. Virol., 147: 2281-2289.

LIM H.J., BAE E.H., LEE Y.J., PARK S.H., LEE K.J., KIM S.R., 2009 - *Detection of Lily symptomless virus, Lily mottle virus, and Cucumber mosaic virus from Lilium grown in Korea by RT-PCR*. - Korean Journal of Microbiology, 45: 251-256.

LIU H.Y., SEARS J.L., MOU B., 2009 - *Spinach (*Spinacia oleracea*) is a new natural host of impatiens necrotic spot virus in California*. - Plant Dis., 93: 673.

LUVISI A., PANATTONI A., MATERAZZI A., 2015 - *Heat treatments for sustainable control of soil viruses*. - Agron. Sustain. Dev., 35: 657-666.

MACKENZIE D.J., MCLEAN M.A., MUKERJI S., GREEN M., 1997 - *Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction*. - Plant Dis., 81: 222-226.

MUMFORD R.A., BARKER I., WOOD K.R., 1994 - *The detection of tomato spotted wilt virus using the polymerase chain reaction*. - J. Virol. Methods, 46: 303-311.

OSMAN F., ROWHANI A., 2006 - *Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan)*. - J. Virol. Methods, 133: 130-136.

PANATTONI A., LUVISI A., TRIOLO E., 2013 - *Elimination of viruses in plants: twenty years of progress*. - Span. J Agric. Res., 11: 173-188.

PARK T.H., HAN I.S., KIM J.B., 2010 - *Review on the development of virus resistant plants in* Alstroemeria. - J. Plant Biotechnol., 37: 370-378.

RIZZO D., MATERAZZI A., STEFANI L., FARINA P., VANARELLI S., PANATTONI A., LUVISI A., 2015 a - *Distribution of regulated viruses in cv. Sangiovese vineyards in Tuscany*. - J. Plant Pathol., 97: 131-135.

RIZZO D., PANATTONI A., STEFANI L., PAOLI M., NESI B., LAZZERESCHI S., VANARELLI S., FARINA P., DELLA BARTOLA M., MATERAZZI A., LUVISI A., 2015 b - *First report of Zantedeschia mild mosaic virus on* Zantedeschia Aethiopica *(L) Spreng in Italy*. - J. Plant Pathol., 92: 399.

RIZZO D., STEFANI L., PAOLI M., TRIOLO E., PANATTONI A., LUVISI A., 2012 - *The sustainability of old grapevine mother plants in relation to new mandatory diagnostic tests for virus control*. - Adv. Hort. Sci., 26(3-4): 148-150.

SPENCE N.J., MILLS P.R., BARBARA D.J., 2000 - *A surbey of viruses of* Alstroemeria *in the UK and the characterization of carlaviruses infecting Alstroemeria*. - Eur. J. Plant Pathol., 106: 843-847.

WEI T., LU G., CLOVER G.R.G., 2009 - *A multiplex RT-PCR for the detection of Potato yellow vein virus, Tobacco rattle virus and Tomato infectious chlorosis virus in potato with a plant internal amplification control*. - Plant Pathol., 58: 203-209.

WEN-CHI H., CHIN-HSING H., SHU-CHUAN L., CHUN-I W., YA-CHUN C., 2010 - *Detection of four calla potyviruses by multiplex RT-PCR using nad5 mRNA as an internal control*. - Eur. J. Plant Pathol., 126: 43-52.