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Refrigerated storage of the fruits of buriti (*Mauritia flexuosa* L.)

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Key words: Brazilian cerrado, buriti, *Mauritia flexuosa*, post-harvest.

Abstract: The objective of this study was to evaluate different storage conditions to maximize the shelf-life of buriti fruits; under ambient conditions the fruits last only 2-3 days. Buriti fruits were stored refrigerated at 10, 12 and 15°C with 85±5% relative humidity, and at room temperature (23±5°C) and 60±5% relative humidity. Fruits were analyzed every three days over a 12-day period for weight loss, respiratory activity, soluble solids, pH, titratable acidity, lipids, protein and fiber. Under the considered conditions, refrigeration at 15°C was found to give the best results.

1. Introduction

Buriti (*Mauritia flexuosa* L.) is a palm tree found from the Atlantic forest to the cerrado (a vast tropical savanna ecoregion) of the Brazilian north, northeast and mid-west in the state of Minas Gerais (Manzi and Coomes, 2009). It also extends to the state of Mato Grosso, as well as Bolivia, Colombia, Ecuador and Peru. There is debate between Brazilian and Peruvian scientists about its origin (Cavalcante, 1991).

Female buriti palm trees produce four to eight infructescences, and each raceme bears 500-2000 fruits (Goulding and Smith, 2007). The fruit is a reddish-brown drupe, with a thin oily yellow-orange pulp that surrounds a relatively large seed (Manzi and Coomes, 2009). The oil contains tocopherols (de França *et al*., 1999; Albuquerque *et al*., 2005), carotenoids (Mariath *et al*., 1989; Silva *et al*., 2009) and pro-vitamin A (Mariath *et al*., 1989; Klemm *et al*., 2008). Moreover, candy made from buriti is an effective treatment of xerophthalmia in children in northeastern Brazil, but the fruit used to make it is not easily preserved. It is good for no more than two to three days under ambient conditions.

The buriti fruit has a hard, red shell that covers an oily pulp that contains carotenoids and ascorbic acid (EMBRAPA, 2007; Silva *et al.*, 2007). The local population collects the fruits when they are released from the mother plant and most of the fruits on the ground are near the desired state of maturity. A 55-kg bunch produces 40 kg of fruits. The local population collects the fruits that they are going to use from the ground.

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Buriti and products made from it are widespread in the Brazilian cerrado. The fruit pulp is used to make a flour for a porridge that helps meet the nutritional requirements of the locals (Almeida and Silva, 1994). The fruit is also a source of vegetable oil, as described by Albuquerque *et al.* (2003). These authors obtained an IR spectrum of the oil that revealed the presence of triolein, the triglyceride of oleic acid, which could be used to control cholesterol in the blood. The oil has been reported to have a relatively high concentration of so-called monounsaturated fatty acids (de França *et al*., 1999), although it really contains fatty acyls that are a part of mono-, di- and triglycerides. That is, the oil had to be hydrolyzed to break down the mono-, di- and triglycerides, forming glycerol plus non-volatile free fatty acids, which are then converted to volatile fatty acid methyl esters and analyzed by gas chromatography (de França *et al*., 1999; AOAC, 2003). In order for the fruits to be used, they must be preserved properly. This is discussed in the next paragraph.

Usually the fruits are collected in a form that is not easily preserved. The palm trees are cut to facilitate the removal of the fruits, drastically reducing the population of buriti trees in the Amazonian region of Peru. This forces the harvesters to travel long distances to gather a significant quantity. The management of buriti via extractavism is small compared to the demands of the regional market (Manzi and Coomes, 2009; Horn *et al*., 2012). Extractavism refers to natural tropical forest areas that are reserved for the extraction of potentially renewable commercial forest products. So, better storage methods are needed to meet the needs of the market and provide social benefits, which are described next.

Buriti offers social benefits to the local population as a source of wealth and employment in the manufacture of products such as licorice, wine, candy, juice and sorbets.

However, these activities have not been given the technical or scientific support needed to make them sustainable and more profitable. While an adequate storage method has not yet been well defined, it is known that the fruit must be scraped off the seeds and dehydrated at room temperature, followed by refrigeration for an undetermined period of time (Almeida and Silva, 1994).

Therefore, the objective of this study was to evaluate different storage conditions for buriti fruits in order to find the best way to maximize shelf-life with attention to the post-harvest behavior of the fruits so as to maintain their quality.

2. Materials and Methods

The fruits used in the study were from the ecological preserve in Jalapão, near the city of Dianópolis in the state of Tocantins at 11°37'40" south latitude and 46°49'14" west longitude at an altitude of 691 m above sea level, where the trees grow in sandy soil. The fruits were picked from the trees when they were 3-4 cm in diameter and dark yellow to brown. The trees were not cut. Due to the height of the tree (10 m), a scaffolding was erected so that the bunches of fruit could be removed with the utmost caution, to prevent damage caused by falling. The bunches were placed in a ventilated polystyrene box to maintain a temperature of 16°C.

After collection, the fruits were sent to the Laboratório de Frutas e Hortaliças do Departamento de Gestão e Tecnologia Agroindustrial da Faculdade de Ciências Agronômicas – UNESP, Botucatu, São Paulo, where they arrived after two days. They were separated randomly into four lots, each containing ten fruits. Three were stored under refrigeration at 10, 12 and 15°C and 85±5% relative humidity. The fourth lot (control) was stored under ambient conditions (23±5°C and 60±5% relative humidity). The following analyses were carried out on ten fruits for each storage condition: weight loss, respiratory activity, soluble solids, titratable acidity, and pH. Analyses were carried out for fruits that were viable for commercialization.

Respiratory activity was determined by the release of CO_2 in each package according to the method of Bleinroth *et al.* (1976), using a saturated solution of barium hydroxide and 0.1 N KOH (0.1 Normal KOH, which is the same as 1 mol/L KOH) and using the formula:

$$TCO_2 = \frac{2.2\ (Vo - V_1)\ 10}{m\ t}$$

where:

TCO_2 is the rate of respiration (mL of CO_2 $Kg^{-1}h^{-1}$);

Vo = mL of HCl needed to titrate the KOH as a standard before the absorption of CO_2;

V_1 = mL of HCl needed to titrate the KOH after the absorption of CO_2;

m = mass of the fruits;

t = respiration time;

2.2 = equivalent weight of CO_2 (44/2), multiplied by the concentration of HCl;

10 = adjustment for the total amount of KOH used.

Soluble solids, pH and titratable acidity were determined by the method of the Instituto Adolf Lutz (IAL, 2008). Soluble solids were measured with a digital Palette PR - 32 refractometer (ATAGO Inc., Bellevue, WA), equipped with automatic temperature compensation. Results were expressed directly in °Brix. The pH was measured with a pH meter and titratable acidity was measured by titrating the acidic fruits with 0.1 mol/L NaOH. The amount of total sugars, lipids, proteins and fibers were determined using the method of Somogyi (1945) and Nelson (1944) by reacting samples with the Somogyi reagent and measuring the absorbance at 535 nm using a Micronal B382 spectrophotometer (Micronal, São Paulo, SP, Brazil). In detail, samples were diluted sufficiently so that an absorbance between 0.2 and 0.8 was produced after reacting 1 mL of neutralized and filtered sample with 1 mL of Somogyi reagent. After putting the samples in a boiling water bath for 10 min, they were cooled to room temperature. Then, 1 mL of the Nelson reagent and 7 mL of water were added. Finally, the absorbance at 535 nm was read. The Somogyi reagent is an arsenomolybdate complex formed by the reaction of ammonium molybdate with sodium arsenate. The Nelson reagent was made of two parts: Part A contained 2.5 g each of Na_2CO_3 and potassium sodium tartrate, 2 g each of $NaHCO_3$ and Na_2SO_4 in 100 mL water. Part B contained 7.5 g $CuSO_4 \cdot 5H_2O$ per 100 mL water, acidified with a drop of conc H_2SO_4. Glucose was used to construct a calibration curve.

Total lipids were determined by performing a 2-h Soxhlet extraction on 3 g of sample using 200 mL of petroleum ether, evaporating off the solvent and weighing the residue. Protein was determined on 0.1 to 0.2 g of sample using the Kjeldahl method using a conversion factor of 6.5 to convert percent nitrogen to percent protein.

The experimental data were analyzed as a 4x5 matrix (temperature x time) by the SISVAR 4.6 program. Averages were evaluated by the Tukey test at 5% probability (Gomes, 1987).

3. Results and Discussion

Beginning on the third day of storage, a weight loss of >10% was found in all storage conditions. The fruits stored at 10 and 23°C had the greatest loss of mass after 12 days. The lowest loss was at 15°C (Table 1). All weight losses were calculated by comparing weights to day zero. Ten fruits were analyzed in each experiment. According to Finger and Vieira (2002) the weight loss of most fresh fruits should be 5-10% to avoid withering or wrinkling. Thus, buriti fruits examined suffered a weight loss that reduced their commercial value.

Buriti fruits demonstrated respiratory behavior that is characteristic of climacteric fruits, as shown in Table 2. According to Chitarra and Chitarra (2005) they are characterized by a rapid increase in respiration and ethylene production during ripening.

The apparent peak at day three of storage was probably due to an adaptation of the fruits to the storage conditions. The fruits that were kept at ambient temperature had their peak respiration on the sixth day of storage, while the fruits stored under other conditions had their peak respiration on the ninth day of storage. The lowest respiratory activity (lowest production of CO_2) was found in fruits stored at 10ºC, i.e. with the lowest production of CO_2. Therefore, a temperature of 15ºC proved to be the most effective for storing the fruits as it resulted in the lowest loss of weight and the latest peak in respiratory activity.

The amounts of soluble solids, pH and titratable acidity are shown in Table 3.

Table 1 - Weights of buriti when stored refrigerated

Temperature	Initial Wt. (g)	3 days (g)	6 days (g)	9 days (g)	12 days (g)
10°C	263.03±7.81 a	238.43±5.74 a	214.50±6.78 a	197.95±3.98 a	191.60±2.45 b
12°C	256.32±6.82 a	237.31±4.81 a	222.10 ±4.91 c	212.56±4.89 c	208.59± 3.99 c
15°C	261.23±5.73 a	245.60±5.97 b	233.65±5.60 d	226.13±6.71 d	223.12± 4.58 d
Ambient	274.75±6.99 b	251.80±6.52 c	229.53±4.57 b	207.65± 5.86 b	194.47±4.98 a

Averages followed by the same lower case letter do not differ significantly from others in the same column and those followed by the same upper case letter do not differ significantly from others in the same row by the Tukey test at 5% probability.

Table 2 - Respiratory activity in buriti fruits stored under refrigeration and 80 ± 5% relative humidity for 12 days

Temperature	Respiratory Activity				
	Days of storage				
	0	3	6	9	12
10°C	24.5±0.64	35.1±1.90	18.5±0.99	35.5±0.42	13.8±0.64
12°C	24.5±0.64	12.1±0.14	17.3±0.42	58.6±2.62	44.2±3.11
15°C	24.5±0.64	31.4±0.35	17.9±0.78	70.9±0.28	36.8±0.64
Ambient	24.5±0.64	54.2±0.28	55.6±0.07	50.8±0.35	-

Averages followed by the same lower case letter do not differ significantly from others in the same column and those followed by the same upper case letter do not differ significantly from others in the same row by the Tukey test at 5% probability.

Table 3 - Soluble solids (°Brix), pH and titratable acidity (g of acid $100g^{-1}$ fresh weight) in buriti fruits under refrigerated storage (80±5% relative humidity) and ambient conditions (23±5ºC and 60±5% relative humidity)

Temperature	Days of storage				
	0	3	6	9	12
			Soluble solids		
10°C	12.37±1.51 aB	14.67±0.58 aAB	16.33±2.52 aA	15.00±1.73 aAB	16.00±0.00 aA
12°C	12.37 ±1.51 aA	14.67±0,58 aA	14.00±0 abA	13.66±0.58 aA	14.67±1.53 aA
15°C	12.37 ±1.51 aB	14.67±1.53 aAB	13.33 ±2.08 bcAB	12.67± 0.58 aB	16.00 ±0.0 aA
Ambient	12.37±1.51 aA	10.33±0,58 bB	10.80±0.00 cB	-	-
C.V. (%)	9.37				
			pH		
10°C	3.83± 0.06 aC	3.77± 0.58 abC	4.33±0.15 aAB	4.57±0.06 aA	4.1±0.00 abB
12°C	3.83 ±0.06aC aBC	3.73±0.58 abC	4.07 ±0.15 bAB	3.93± 0.23 bBC	4.23±0.25 aA
15°C	3.83± 0.06aC aBC	3.67±1.53 bC	4.13±0.06 abA	4.00±0.10 bAB	3.97±0.12b AB
Ambient	3.83±0.06aC aB	3.90±0.58 aAB	4.10 ±0.0 bA	-	-
C.V. (%)	2.63				
			Titratable acidity		
10°C	0.68±0.06 aB	0.84±0.02 aA	0.41±0.02 aC	0.47±0.07 bC	0.60±0.00 Ab
12°C	0.68 ±0.06 aB	0.84±0.02 aA	0.51 ±0.03 aC	0.60 ±0.04 aBC	0.52±0.05 aC
15°C	0.68 ±0.06 aAB	0.76 ±0.02 aA	0.49±0.02 aC	0.58±0.04 abBC	0.60 ±0.01 aAB
Ambient	0.68± 0.06 aA	0.63±0.02 bA	0.42±0.00 aB	-	-
C.V. (%)	9.54				

Averages followed by the same lower case letter do not differ significantly from others in the same column and those followed by the same upper case letter do not differ significantly from others in the same row by the Tukey test at 5% probability.

The amount of soluble solids did not show a significant difference when stored at 10, 12 or 15°C. The fruits stored under ambient conditions exhibited a decrease in soluble solids on day three, but it dropped no further on day six. On the ninth day, contamination by the fungus *Monilinia fructicola* was observed and this made the fruits unsuitable for consumption. The fruits stored under ambient conditions maintained a relatively low amount of soluble solids during the experimental period, possibly due to the lower respiratory rate compared to refrigerated storage. The small increase in the concentration of soluble solids at the end of the experiment could be related to the weight loss.

On the third day of storage, there was a tendency for the pH to increase, as shown in Table 3. In the case of ambient storage, this can be due to the process of senescence (Chitarra and Chitarra, 2005). The increase in pH was seen throughout the experiment. When stored at 10°C, the pH was nearly constant on day three, but increased on days six and nine, followed by a decrease on day 12. When stored under ambient conditions, the pH increased until the last day of analysis.

The titratable acidity increased on day three under all storage conditions, followed by a decrease on subsequent days. Ambient storage presented the lowest amount of titratable acidity, probably due to infestation by pathogens that consumed acid in their metabolism (Chitarra and Chitarra, 2005; Özcan and Haciseferogullari, 2007).

The data on total sugars, lipids and protein are presented in Table 4.

Albuquerque *et al*. (2005) reported levels of total sugars in buriti that varied by about 2.10%, similar to what we found. Moreover, Hiane *et al.* (1992) reported values of 11.36% ± 1.81, which are higher than those found in the present experiment.

The amount of total sugars increased from day zero to the third day of storage, followed by a decrease on the sixth day and increases on subsequent days. Carbohydrates are oxidized by the respiratory process (Chitarra and Chitarra, 2005; Rodriguez-Guisado *et al*., 2009), causing the decrease. The increase in concentration on later days was probably related to the loss of weight.

According to Cavalcante (1991), buriti fruit contains a relatively large amount of lipids, which are an important source of energy. This was also reported by de França *et al*. (1999), Albuquerque *et al*. (2005), and Silva *et al*. (2009), Rodrigues *et al.* (2010). However, all these authors reported finding free fatty acids, when they were most likely fatty acyls as a part of mono-, di- and triglycerides.

Table 4 - Amounts of total sugars, lipids, protein and fiber (%) in buriti fruits under refrigerated storage (80±5% relative humidity) and ambient conditions (23±5ºC and 60±5% relative humidity) for 12 days

Temperature	Storage Days				
	0	3	6	9	12
			Total sugars		
10°C	2.22 aB	3.25 aA	0.91 aC	1.29 bC	2.72 aAB
12°C	2.22 aB	2.93 aA	0.80 aC	2.69 aAB	2.42 aAB
15°C	2.22 aB	3.09 aA	0.83 aC	2.40 aB	2.68 aAB
Ambient	2.22 aB	3.07 aA	0.96 aC	-	-
C.V. (%)	12.64				
			Lipids		
10°C	14.00 aC	18.67 aB	18.53 aB	14.80 cC	21.00 aA
12°C	14.00 aC	18.13 aB	17.30 abB	18.23 aB	21.33 aA
15°C	14.00 aC	15.67 bB	16.93 abB	16.70 bB	20.47 aA
Ambient	14.00 aB	13.30 cB	18.00 bA	-	-
C.V. (%)	3.65				
			Protein		
10°C	0.26 aD	0.26 aB	0.35 aB	0.37 aC	0.33 aA
12°C	0.26 aC	0.25 aB	0.21 cB	0.29 bB	0.31 bA
15°C	0.26 aB	0.21 bB	0.35 aB	0.27 bB	0.26 cA
Ambient	0.26 aB	0.22 bB	0.29 bA	-	-
C.V. (%)	2.75				
			Fiber		
10°C	10.43 aC	10.73 aBC	10.10 bC	11.30 aB	13.00 aA
12°C	10.43 aC	9.60 bD	10.37 abCD	11.70 aB	12.67 aA
15°C	10.43 aC	8.50 cD	10.90 aC	11.53 aAB	11.83 bA
Ambient	10.43 aA	8.70 cB	10.50 abA	-	-
C.V. (%)	3.37				

Averages followed by the same lower case letter do not differ significantly from others in the same column and those followed by the same upper case letter do not differ significantly from others in the same row by the Tukey test at 5% probability.

This is a common mistake which is made when fatty acyl amounts are determined by gas chromatography (GC) only after hydrolyzing the glycerides. This forms free fatty acids, which are then esterified to form fatty acid methyl esters (FAMEs) that are volatile enough to be analyzed by GC. Therefore, a clever marketing or sales representative from producers of a competing company could say that all these studies demonstrate that buriti fruits rapidly turn rancid, since free fatty acids were supposedly found. When oils (glycerides) turn rancid, it is due to partial hydrolysis of glycerides to form malodorous free fatty acids. The article by Silva *et al*. (2009) is especially confusing, since it reports actually finding 3.1% free fatty acids in buriti oil, based on their separation by size exclusion chromatography: it is not clear whether the authors really meant free fatty acids or fatty acyls that are part of triglycerides. They go on to report the profile of free fatty acids, but it is based on GC analysis of FAMEs, so it refers to the fatty acyl profile of mono-, di- and triglycerides. Vegetable oils contain mono-, di- and triglycerides, and therefore the analysis method requires that they be hydrolyzed into free fatty acids and glycerol, followed by forming volatile fatty acid methyl esters (FAMEs), which can be analyzed by gas chromatography (Mannina *et al*., 1999; AOAC, 2003). It is not clear whether the 3.1% free fatty acids separated by size exclusion chromatography were part of glycerides or if they were truly free fatty acids, caused by the oil turning rancid.

However, the present experiment truly measured total lipids and found more than the 2.5 to 5.5% reported by Hiane *et al.* (1992) and Donadio *et al*. (2002). Albuquerque *et al*. (2005) reported finding 11.24% lipids. Carneiro and Carneiro (2011) found 18.16% lipids in buriti pulp.

The amount of lipids increased starting on the third day of storage and stayed almost constant throughout the 12-day experiment (Table 4). On the third day, the fruits stored at 10 and 12°C showed no significant differences, but more lipids were found when stored at 15°C. The fruits stored under ambient conditions had fewer lipids that the fruits stored under refrigeration. On day 12 there were no significant differences in lipid content in the refrigerated samples. The increase in lipid concentration with time could be related to the weight loss that occurred.

According to Donadio *et al*. (2002) buriti fruits had about 2.3 to 5.5% protein, while Hiane *et al*. (1992) found 2.12%, Carneiro and Carneiro (2011) found 1.30% and Darnet *et al*. (2011) found 3.7%. In the present study, there were no significant differences in protein concentrations in fruits stored at 10 and 12°C, but these values were higher than those of fruits stored at 15°C and under ambient conditions, which were not statistically different from each other. On the sixth day of storage, there was an increase in protein concentration, with the exception of those stored at 12°C. On the ninth day, the concentration of protein increased in the fruits stored at 10 and 12°C, while those stored at 15°C showed a decrease.

Donadio *et al*. (2002) found that the fiber content in buriti fruits varied from 10.4 to 27.5%. Hiane *et al*. (1992) reported about 12.31% fiber. Darnet *et al*. (2011) found 22.8% dietary fiber in buriti fruits from the Amazon. The concentrations of fiber found in the current study ranged from 8.5 to 13.0%: on the third day, the fruits stored at 10°C had the highest concentration of fiber, followed by those stored at 12°C; those stored at 15°C and under ambient conditions had the lowest concentrations and were not significantly different from each other. Starting on the third day there was an increase in the concentration of fiber which then continued slowly throughout the 12-day experiment. The increase could be simply due to the loss of weight, so the total amounts were about the same.

4. Conclusions

Refrigeration was effective in extending the shelf-life of buriti fruits, increasing it by at least three days. The data presented regarding the decrease in weight and respiratory activity demonstrate that a temperature of 15°C was the most effective in maintaining the quality of buriti fruits.

This work should not be taken as reflecting FDA policy or regulation.

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Short note

Biochemical and physiological adjustments in common Bermudagrass (*Cynodon dactylon* [L.] Pers.) and tall Fescue (*Festuca arundinacea* Schreb.) under low temperature stress

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Key words: Antioxidant enzymes activity, common Bermudagrass, low temperature stress, tall fescue, Turfgrasses.

Abstract: Low temperature is a restrictive factor for turfgrass growth and development in temperate regions. A study was conducted with the purpose of examining the physiological and antioxidant response of two turf species, *Festuca arundinacea* Schreb. 'Starlett' and *Cynodon dactylon* [L.] Pers. 'California Origin' to cold stress in a growth chamber at the College of Agriculture, Shiraz University. Five temperatures (25, 15, 7.5, 0 and -7.5°C) in four replicates were examined in a completely randomized design experiment. It was revealed that under low temperature stress, soluble sugar contents, proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) were increased in both turfgrasses. Antioxidant enzyme activity, particularly catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1), was increased as a result of temperature reduction from 25°C to 0°C. Tall fescue is thought to be better adapted to cold stress than common bermudagrass due to higher soluble sugar contents, proline, malondialdehyde and antioxidant enzyme activity. The results show that scavenging enzymes have a direct effect in cold season tolerance of turfgrass and improve the defense mechanism of plants, but their exact role merits further investigation.

1. Introduction

Temperature is a limiting element for distribution of plants worldwide (Sakai and Larcher, 1987). Cold season turfgrasses such as *Festuca* in temperate regions have a good adaptability to low temperature, but under severe winter conditions may suffer considerable damage (Levitt, 1980). Although fescues are cultivated in transition zones, warm season turfgrasses such as bermudagrass are preferred (Carrow, 1994). Plant responses to cold stress and subsequent adaptation occurs at physiological and biochemical levels, as well as cellular and molecular extents (Gulzar *et al*., 2011). Harsh low temperatures result in oxidative stress and a change in proline and sugar content of the cells. The vital means for interaction of plants to these stresses is a balance between antioxidant enzymes and reactive oxygen species (ROS). The damaging ROS responsible for oxidative stress consists of free radicals: superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$) and other molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gill and Tuteja, 2010). The precise role of antioxidant enzymes which give tolerance to cold stress in turfgrasses has not yet been investigated, but their relieving effect to other oxidative stresses has been reported by other researchers (Jiang *et al*., 2005). Rogers *et al*. (1975) examined the proline amount of *Zoysia japonica* Steud. 'Meyer' during the months from October to March and found that there is an increase in proline from October to December. It has been shown that for the period of adaptation to cold, SOD and CAT activity in *Agrostis stolonifera* L., *Poa pratensis* L. and *Lolium perenne* L. significantly increased (Sarkar and Bhowmik, 2009).

The main objective of the present study was to investigate the effects of low temperature stress on biochemical and physiological responses of tall fescue and common bermudagrass. To the best of our knowledge, this is the first report on how these turfgrasses counter cold stress.

2. Materials and Methods

Plant materials and experimental conditions

The experiment was conducted in a growth chamber (Gallenkamp, Germany) at the Department of Horticultural Sciences at the College of Agriculture, Shiraz University (29°36' N and 52°32' E, elevation 1810 m). Seeds of common bermudagrass (*Cynodon dactylon* [L.] Pers. 'California origin') and tall fescue (*Festuca arundinacea* Schreb. 'Starlett') were cultivated in 5 L plastic pots containing a

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mixture of 1:2 (v/v) of loamy soil/decomposed farmyard manure. Irrigation was carried out on a daily schedule. Established turfs were clipped from 3 cm above ground by a hand mower and were transferred to the growth chambers prior to the application of treatments. All treatments received a constant light intensity of 3000 Lux, relative humidity of 65±5% and a 12 h photoperiod. Low temperatures were maintained at 25, 15, 7.5, 0 and -7.5°C for 48 h.

Experimental design and data analysis

Experiment factors were arranged in a completely randomized design with four replications. Data were analyzed using SAS software (ver. 9.1.3) and means were compared using the least significant difference (LSD) test at $p<0.05$.

Reducing sugars and proline content

Phenol-sulfuric acid reactions were used to determine the reducing sugar content. Shoot samples were oven dried at 60°C for 48 h and then ground to a fine powder using an electric mill. Samples (0.2 g) were diluted with 80% ethanol and centrifuged at 13500 rpm. Supernatant was further diluted to 25 ml by 80% ethanol. Then, 1 ml of extract was mixed with 1 ml of 5% phenol. Five ml of concentrated sulfuric acid were added to tubes and immediately stirred. Light absorption was measured by a spectrophotometer (Biochrome, UK) at 490 nm wavelength (Dubois *et al*., 1956). Proline was determined according to the method used by Bates *et al*. (1973) using a spectrophotometer at 520 nm wavelength.

Measurement of antioxidant enzyme activity

To extract antioxidant enzymes, fresh leaf samples (0.5 g) were collected and ground to a fine powder in a mortar by adding liquid nitrogen and then homogenized with an ice cold enzyme extraction buffer containing 0.5% polyvinylpyrrolidone (PVP), 3 mM EDTA, and 0.1 M potassium phosphate buffer (pH=7.5). The extracted samples were centrifuged for 10 min at 13500 rpm and 2-4°C and stored on ice until used. The resulting supernatants were used for enzyme analysis. CAT activity was determined according to the procedure used by Dhindsa *et al*. (1981) and SOD activity was determined as described by Beauchamp and Fridovich (1971).

Malondialdehyde (MDA)

As for H_2O_2, 0.25 g of leaf samples were ground in a mortar containing 5 ml TCA (0.1%). Leaf extracts were centrifuged at 10000 rpm for 5 min. Supernatants (250 µl) were mixed with 1 ml MDA solution containing 20% TCA and 0.5% thiobarbituric acid. The mixtures were warmed at 95°C for 30 min and then immediately cooled on ice. Sample tubes were centrifuged at 10000 rpm for 10 min. Absorption of light was measured by a spectrophotometer at 532 nm wavelength according to Heath and Packer (1969).

Hydrogen peroxide (H_2O_2)

Leaf samples (0.25 g) were ground in a mortar containing 5 ml trichloroacetic acid (TCA) (0.1%). Extracts were centrifuged at 10000 rpm for 5 min. Supernatants (250 µl) were mixed with 250 µl phosphate buffer (100 mM) and 500 µl potassium iodide (1 M). Absorption of light was measured by a spectrophotometer at 390 nm wavelength according to Alexieva *et al*. (2001).

3. Results

With a decrease of temperature from 25°C to -7.5°C, reducing sugars increased considerably, with tall fescue showing a greater increase than common bermudagrass. The highest soluble sugar content in tall fescue was formed at -7.5°C and the highest reducing sugar content produced in common bermudagrass was detected at 0°C (Table 1). There was no significant difference between the turfgrasses for proline content. The highest proline content was observed at 0°C and the lowest proline content was seen at 25°C. The interaction of temperature and turf species showed that tall fescue at 25°C had the lowest proline content, while tall fescue at -7.5°C had the highest (Table 1). It was found that as temperature decreased from 25°C to 7.5°C, CAT activity increased.

The greatest CAT activity was observed in tall fescue at 7.5°C and the least was seen in bermudagrass at -7.5°C (Table 1). Comparison of the means showed that SOD activity in tall fescue is greater, but not significantly different from common bermudagrass. Maximum SOD activity in bermudagrass was detected at 0°C, while the minimum was found at 25°C in tall fescue. As the temperature diminished from 25°C to -7.5°C, MDA amassed continuously in the plants. MDA accumulated significantly more in common bermudagrass with the highest amount built up at -7.5°C (Table 2). H_2O_2 increased in plants as the temperature lowered to 0°C. The most H_2O_2 was produced in common bermudagrass at 0°C, whilst the lowest was observed in tall fescue at 25°C (Table 2).

4. Discussion and Conclusions

As the temperature decreased from 25°C to -7.5°C, soluble sugars and proline content increased, which tall fescue had higher amounts at -7.5°C (Table 1). A similar behavior was found in saltgrass (*Distichlis spicata* L.), centipedegrass (*Eremochloa ophiuroides* [Munro]), annual bluegrass (*Poa annua* L.) and buffalograss (*Bouteloua dactyloides* [Nutt.]) (Fry, 1993; Shahba *et al*., 2003). Generally, one of the first reactions by these plants to counter the chilling stress of winter is a buildup of sugar (Fry, 1993; Ball *et al*., 2002), whilst amino acids help adapt the plants to low temperature (Guy, 1990). Proline and reducing sugars serve as cryoprotectants through increasing the concentration of cell content and reducing the water potential (Ball *et al*., 2002). Comparable results were observed in zoysiagrass (*Zoysia japonica* Steud.) and annual bluegrass (Dionne *et al*., 2001).

Table 1 - Effects of cold stress on biochemical changes [reducing sugar, proline content, catalase (CAT) and superoxide dismutase (SOD) activity] in the two turfgrasses used in this study

Turfgrass	Temperature (°C)					Mean
	-7.5	0	+7.5	+15	+25	
Reducing sugar (mg·g-¹ d.w.)						
Tall fescue	176.4±60.1 a	160.8±16.3 ab	130.2±27.3 bcd	114.8±13.2 cd	104.3±22.4 d[z]	137.4 A
Bermudagrass	121.9±11.5 bcd	158.9±21.0 abc	140.9±18.7 a-d	117.3±19.8 bcd	96.5±14.7 d	127.1 A
Mean	149.2 A	159.8 A	135.5 AB	116.1 BC	100.4 C	
Proline content (µg·g-¹ d.w.)						
Tall fescue	35.8±3.7 a	35.1±3.4 a	27.2±2.7 b	13.7±3.1 cd	9.2±0.9 d	24.2 A
Bermudagrass	26.4±1.7 b	31.8±1.5 ab	27.3±5.1 b	17.3±4.5 c	12.7±1.3 cd	23.1 A
Mean	31.1 A	33.5 A	27.2 B	15.5 C	11.0 D	
CAT (U g·g-¹ d.w.)						
Tall fescue	36.3±5.4 c	46.7±6.1 ab	52.8±9.8 a	37.5±3.6 c	32.8±5.7 c	41.2 A
Bermudagrass	31.5±4.5 c	39.5±4.4 bc	47.5±2.9 ab	41.5±3.9 bc	36.9±5.8 c	39.3 A
Mean	35.4 C	43.1 B	50.1 A	39.5 BC	34.8 C	
SOD (U g·g-¹ d.w.)						
Tall fescue	179.6±17.6 abc	161.6±33.1 a-d	186.6±30.5 ab	126.0±19.0 de	106.6±17.0 e	152.1 A
Bermudagrass	127.3±23.1 cde	193.3±25.1 a	145.0±42.7 a-e	137.6±58.6 b-e	120.6±11.7 de	144.8 A
Mean	153.5 AB	177.5 A	165.8 AB	131.8 BC	113.6 C	

[z] In each variable, data followed by the same letters±SD (small letters for interactions and capital letters for means) are not significantly different at 5% level of probability using LSD test.

Table 2 - Effects of cold stress on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the two turfgrasses used in this study

Turfgrass	Temperature (°C)					Mean
	-7.5	0	+7.5	+15	+25	
MDA (µmol g-¹ f.w.)						
Tall fescue	7.5±1.6 cd	7.8±1.3 bc	6.8±0.8 cde	4.4±0.7 fg	3.9±0.7 g[z]	6.1 B
Bermudagrass	11.6±0.7 a	9.5±0.7 b	7.7±0.8 c	5.9±1.0 def	5.1±0.7 efg	8.0 A
Mean	9.6 A	8.6 A	7.3 B	5.1 C	4.5 C	
H_2O_2 (µmol g-¹ f.w.)						
Tall fescue	5.1±0.9 a	4.7±0.7 a	3.5±0.6 b	2.9±0.4 b	2.7±0.4 b	3.8 B
Bermudagrass	5.0±0.3 a	5.3±0.4 a	4.9±0.7 a	3.5±0.5 b	3.1±0.7 b	4.3 A
Mean	5.0 A	5.0 A	4.2 B	3.2 C	2.9 C	

[(z)] In each variable, data followed by the same letter ± SD (small letters for interactions and capital letters for means) are not significantly different at 5% level of probability using LSD test.

The increase in CAT and SOD activity found in this study is assumed to protect the cells from oxidative damage caused by cold stress as seen in other plants (Matsumura *et al*., 2002; Larkindale and Huang, 2004; Jiang *et al*., 2011). SOD converts superoxide ($O_2^{\cdot-}$) to H_2O_2 and CAT detoxifies the latter to water and oxygen (Fuchs *et al*., 1997; Polidoros and Scandalios, 1999). Antioxidant enzymes help maintain cell homeostasis under severe low temperatures by scavenging as well as signaling, although their definite function should be further elucidated (Polle, 1997). Higher antioxidant enzyme activity in tall fescue could be attributed to better cold tolerance compared to common bermudagrass. MDA and H_2O_2 increase in the turfgrasses in this research is dependent on the cold stress received (Table 2). MDA and H_2O_2 are produced by lipid peroxidation of plants under chilling stress (Leshem, 1987; Wise and Naylor, 1987). These two sensitive indicators are considered to point toward the extent of low temperature stress and damage inflicted to the plant (Xu *et al*., 2006). Greater amounts of these two substances in common bermudagrass compared to tall fescue could be interpreted as a greater sensitivity to and injury from low temperatures (Table 2), which is consistent with the reports in Manila grass (*Zoysia matrella* L.) (Wang *et al*., 2009).

Overall, cold stress produces large amounts of ROS which causes oxidative damage to plants through vast destruction of proteins, carbohydrates, lipids, cellular membranes, DNA and major decline of ATP reserve, and finally cell death (Dionne *et al*., 2001; Gill and Tuteja, 2010). Since ROS has multifunctional roles, it is essential for the cells to control the level of ROS tightly to avoid any oxidative injury and not to eliminate them

entirely (Sharma *et al*., 2012). It is concluded that both turfgrasses increase reducing sugars, proline, CAT, SOD, MDA and H_2O_2 in response to lower temperatures, but tall fescue has a better defense mechanism than common bermudagrass and is more tolerant to cold stress. The results show that scavenging enzymes have a direct effect in cold season tolerance of turfgrass and improve the defense mechanism of plants, but their exact role merits further investigation.

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The relationship between grape phylloxera and *Fusarium* root infection

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Key words: *Fusarium ssp*., grape phylloxera, grape root.

Abstract: Phyloxera seems to have key role in the fungal pathogen infection ratio while the fungal spread reduces the ability of pheloxera to reproduce. Intact roots of four-month-old grape plants were inoculated with phylloxera eggs in presence or absence of fungal pathogens. *Fusarium solani SY7* infection was detected in all plant parts when grapevine roots were infested with phylloxera. The spread ratio of *Fusarium solani SY7* increased from 74 to 100% of the infested plants with phylloxera. On the other hand, the phylloxera on *F. solani SY7* infected roots were developed more slowly, since the nymphs and tuberosities were significantly decreased 49% and 31% respectively. The total plant biomass decreased to 29% in the presence of both *F. solani SY 7* and phylloxera as compared to 9 and 17% in the presence of *F. solani SY7* or phylloxera, respectively. This study sheds light on the correlation between fungi, phylloxera and grapevine and could help in the application of integrated pest management (IPM) programs against grape phylloxera.

1. Introduction

Grapevine phylloxera (*Daktulosphaira vitifoliae Fitch*) is considered the most destructive grapevine insect pest in Syria where more than 70,000 ha of this crop, annually producing approximately 540,000 t of grapes, are planted (Makee *et al.*, 2010). Grape phylloxera invaded Syria on nursery stock from bordering countries during the 1920s where it quickly spread in Syrian vineyards among the non-resistant local *Vitis vinifera* L. grape (Makee *et al.*, 2003).

In Syria, the most commonly used resistant rootstocks are Ru140 (*V. rupestris* x *V. berlandieri*), R99 (*V. rupestris* x *V. berlandieri*), 3309C (*V. riparia Michaux* x *V. rupestris*) and 41 B (*V. vinifera* x *V. Berlandieri* Planchon). However, local phylloxera demonstrate an ability to develop and reproduce on all American rootstocks (Makee *et al*., 2010). In addition, the susceptibility of Ru140 rootstock was found to be higher than that of R99 and 3309C rootstocks (Makee *et al*., 2003). The rootstocks often allow limited growth and reproduction of phylloxera, but they may also reduce the growth vigor of grafted varieties because of incompatibility phenomena. Moreover, most Syrian grapevines are planted in areas of 300-500 mm rainfall, thus they are unirrigated and such conditions are inappropriate for resistant rootstocks (Makee *et al*., 2010).

Granett *et al*. (1996) reported that once a vineyard is infested with grape phylloxera it is expected to cease its production in about two to five years. Generally, spring phylloxera is found primarily on the small feeder roots that proliferate during root flush. However, during summer when the climate is dry and the top of the plant is growing, phylloxera is found extensively on the infected mature older roots. In winter, phylloxera is found on mature roots due to the disappearance of feeder roots (Omer *et al*., 1997). Once the phylloxera's proboscis is inserted into root bark cells, the parenchyma cells below the suberized outer layer, they inject saliva that, in turn, trigger the root cells to increase in size and number. These galls provide a feeding site from which stored nutrients such as sugars and amino acids can be extracted by developing phylloxera, which leads to a decrease in vine vigor and eventually destroys the roots causing vine death (Omer *et al*., 2002). The injured grapevines are usually attacked by secondary soil-borne pathogens at the insect feeding site (Omer and Granett, 2000). Granett *et al*. (1998) and Lotter *et al*. (1999) revealed that fungal infections associated with pylloxera galls are pathogenic and cause root death. In addition, fungal invasion of the roots at grape phylloxera feeding sites cause severe infections in grapevines. *Fusarium* is a large genus of filamentous fungi, and most *Fusarium* species are harmless saprobes and relatively abundant members of the soil microbial community (Domsch *et at*., 1980; Nwanma *et al*.,1993). This ecological habitat of the fungus implies that *Fusarium* could be a useful resource of extra cellular enzymes. The fungal isolate *F. solani SY7* was the best xylanase producer among tested isolates (Arabi *et al*., 2011; Bakri *et al*., 2012). Vine varieties susceptible to grape phylloxera were also highly susceptible to *F. oxysporum* (Omer *et al*., 1995; 1999). Granett *et al*.

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(1998) also demonstrated that the two root types with *V. vinifera* parentage, Carignane and AXR#1, which are most susceptible to phylloxera feeding, are also susceptible to infection by *F. oxysporum* pathogen.

The present study therefore aims to investigate whether the presence or absence of phylloxera could enhance *F. solani SY7* fungal infection in the local Balady *V. vinifera* variety vine roots and the consequences of the interaction between the grape phylloxera and *F. solan SY7* on the vine. In this context, the interaction between root infection and the three *Fusarium* species root and root- feeding grape phylloxera were first investigated under controlled conditions, then the effects of fungal infection with and without the presence of phylloxera on grape vine vigor (plant biomass, roots, branches and leaves, internodes weights) were determined. The process included identification of the *Fusarium* species that invade the roots from grape phylloxera feeding sites.

2. Materials and Methods

Establishment of the phylloxera colony

Grape phylloxera was originally collected from field-infested roots of the local grape varieties in southern parts of Syria. The phylloxera colony was established according to the procedures mentioned by De Benedictis and Granett (1993). Fresh and healthy pieces of local grape variety (Makee *et al*., 2008) "Balady" roots, 4-7 mm in diameter and 5-7 cm long, were taken and washed with tap water. Each piece was wrapped with moist cotton wool at one end, and then 10 to 15 phylloxera eggs were placed on each piece. The infested root pieces were placed on a wet filter paper disk inside plastic Petri dishes (12 cm diameter and about 1 cm deep, three to four root pieces per dish). For ventilation purposes, the Petri dish lid was modified with a 1-1.5 cm cloth–screened hole. Dish edges were sealed with parafilm, kept in plastic boxes with tightly fitting lids and incubated at 25°C in the dark with 75% relative humidity. The root pieces were replaced when they desiccated rotted or the phylloxera became crowded.

Potted inoculation procedure

Before inoculation, three-day-old eggs (n =100) were removed from the colony and placed in 1.5 ml plastic tubes for surface sterilization. One ml of formaldehyde was added to the eggs and the tubes were gently shaken for 10 min. The sterilizing solution was removed from the mix and the eggs were extracted and placed on a sterile filter paper and dried for 5 min. They were then kept in a Petri dish, sealed with parafilm to prevent contamination and the escape of the phylloxera crawlers. Egg sterilization was carried out under sterile conditions (Makee *et al.*, 2003).

Fungal isolates

The fungal isolates were obtained from the plant pathology laboratory of the Atomic Energy Commission of Syria (Arabi *et al*., 2011) (Table 1). Host plant root samples infested with *Fusarium* were collected from different locations in Syria. Roots were sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, roots were dipped in 70% Ethanol for 1 min and then washed once with distilled water. Roots were cut into small slides under sterile conditions and transferred to Petri dishes containing potato dextrose agar (38g/L) (PDA, DIFCO, DETROIT, MI, USA) (Alazem, 2007). Thirteen mg/1 Kanamycin sulphyate were added after autoclaving and 10 days incubation at 23 ± 1°C in the dark to allow mycelia growth. All isolates were identified morphologically, according to Nelson *et al*. (1993). Emphasis was placed on selecting isolates that induced differential reactions on specific genotype, pathogencity and *in vitro* xylanase activity (Alazem, 2007; Arabi *et al*., 2011; Bakri *et al*., 2012). The above mentioned parameters lead to select of three monosporic isolates *F. culnorum SY*3*, F. solani SY*7 *and F. equisesti SY*24. The *Fusarium* isolates used in this study, their location, year of collection and xylanase production are listed in Table 1 (Arabi *et al*., 2011). The cultures were maintained on silica gel at 4°C until needed. Eighty "Balady" grape stem pieces were dipped in a solution of 2000 ppm IAA (Indol Butric Acid) for 2 min, then planted in plastic pots contacting sterile moistened soil. Finally, they transferred to 10-L plastics pots, after four months. The experiment was conducted in greenhouse, using a randomized complete block design with four replicates of four plants for each of the following treatments in each treatment.

Table 1 - *Fusarium* isolates, location, year of collection and extra cellular xylanase production in solid state fermentation after five days of inoculation at 30°C

Isolate	Location of Syria	Year of collection	Xylanase (U/G)
F. culnorum *SY3*	North-West	2005	163.69
F. solani *SY7*	Middle-Region	2003	908.2
F. equiseti *SY24*	North-East	2005	122.43

1 - Infection with phylloxera: roots of vines were infected with phylloxera eggs (50 eggs/root).

2 - Infection with *Fusarium spp*: vine roots were dipped in a fungal solution (5 x 10^4 spores/ml) containing an equal mix of spores (*F. culnorum SY*3*, F. solani SY*7*, F. equiseti SY*24) for 15 s, then dried for 30 min.

3 - Infection with *Fusarium spp* and phylloxera: vine roots were dipped in a fungal solution (5 x 10^4 spores/ml) containing an equal mix of spores (*F. culnorum SY*3 *, F. solani SY*7*, F. equiseti SY*24) for 15 s, dried for 30 min, and then infected randomly by phylloxera eggs.

4 - Plant control (free of *Fusarium spp.* and phylloxera): plants were transplanted into 10-L pots. Each experimental unit consisted of two plants. Pots were filled with sterile soil. The pots were all placed in a greenhouse at 25 ± 1°C (day) and 23±1°C (night) with 16-h

daylight and 85-95% relative humidity. Plants were irrigated with water as needed.

A year later, the following parameters were measured: plant biomass, root weight, root number, internode weight. Five root pieces of each tested plant were sampled. Microscopic inspection was performed to determine the number of tuberosities and feeding nymphs for each tested plant.

Fungal inspection

Plant samples (roots, leaves and branches) were collected from each tested plant, surface sterilized for 3 min in 5% NaOCl, and rinsed twice in distilled water. Six disks to each roots, leaves and branches were transferred to Petri dishes containing PDA with 13 mg/L Kanamycin sulphyate added after autoclaving and incubated for 4 weeks at 23 + 1°C in the dark to allow mycelia growth. Mycelia edges of *F. culnorum SY*3*, F. solani SY*7*,* and *F. equiseti SY*24 were identified morphologically as describe by Nelson *et al.* (1993). The infection percentage of fungal ratio was calculated using the formula $R = F_e / Ft \times 100$, where R = the percentage of fungal ratio, F_e = number of disks that contain the fungi from roots, leaves and branches, and Ft = number of the total disks with or without fungi from roots, leaves and branches.

Statistical analysis was performed using the STATISTIC program version 6 (Statsoft, Inc. 2003) at 5% level ($P = 0.05$). Means were subjected to analysis of variance tested for significance using Tukey HSD test.

3. Results

Incidence of Fusarium spp infection in different parts of plant with or without phylloxera

The results demonstrated that fungal infection ratio in the whole plant was 74, 1.7 and 0% of *F. solani SY*7*, F. equiseti SY*24 and *F. culmorum SY*3, respectively. Furthermore, the ratio of infection with *F. solani SY*7 in treatment 4 increased 26% to reach 100% (Table 2).

Effect of different treatments on plant

The plant biomass (Fig. 1, I) decreased significantly in the presence of phylloxera and *Fusarium solani SY*7 (9 and 17%, respectively). This effect is more evidenced in the presence of fungi and phylloxera 29% (F=2; df=1, 3; p<0.05). Vine roots infection with *Fusarium spp*. had no effect on root weight (Fig. 1, II) compared with the control, while infection with phylloxera increased 36% significantly comparing to control (F=2; df=1, 3; p<0.05).

Branch and leaf weight (Fig. 1, III) decreased significantly (F=2; df=1, 3; p<0.05) compared to the control in the presence of phylloxera and *F. solani SY*7 (53% and 31%, respectively). This effect is more evidenced in the presence of phylloxera with infection of *F. solani SY*7 58%. The average internode weight (Fig.1, IV) in treated plants with both phylloxera and fungi increased considerably (50%) as compared to the control. However, no obvious correlation between phylloxera and fungi concerning the internode weights was shown in the experiment. In addition, the root number of vines exposed to the fungus was notably decreased but to a lesser degree compared to plants infested with phylloxera or both fungi and phylloxera (F=3.6; df=1, 3; p<0.05) (Fig. 2).

Effects of F. solani SY7 infection on nymph and tuberosity numbers

The numbers of nymphs and tuberosities in vines infested with phylloxera decreased significantly (F=85.3; df=1.1; p<0.05), (F20=; df=1.1; p<0.05) in comparison with vines infested with both phylloxera and fungi. In addition, nymphs and tuberosities were reduced by 49 and 31%, respectively, compared to the control plants (Table 3).

4. Discussion and Conclusions

Phylloxera seems to have key a role in the fungal pathogen infection ratio and, in contrast, the fungal spread reduces the ability of pheloxera to reproduce. The high infection ratio of *F. solani SY7* (74%) may attribute to its ability to spread in the phloem parenchyma through a special mechanism which allows the fungi to infect the roots (Omer *et al*., 1999). Phylloxera can serve as a vector and transport fungal propagates from infected to healthy roots (Omer *et al*., 2000). Therefore, our data suggest that the injury caused by phylloxera may give benefit to *F. solani*

Table 2 - Incidence of *Fusarium spp.* infection in different parts of plant with or without phylloxera

Treatment	*F. solani SY7 %*			*F. equiseti SY24 %*			*F. culnorum SY3 %*		
	Roots	Branches	Leaves	Roots	Branches	Leaves	Roots	Branches	Leaves
Phylloxera	0	0	0	0	0	0	0	0	0
Fusarium mix	72	77	75	0	0	0	0	0	0
Phylloxera + *Fusarium* mix	100	100	100	5	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0

Data represents the infection percentage of *Fusarium spp.* ratio in roots, branches and leaves in each treatment.

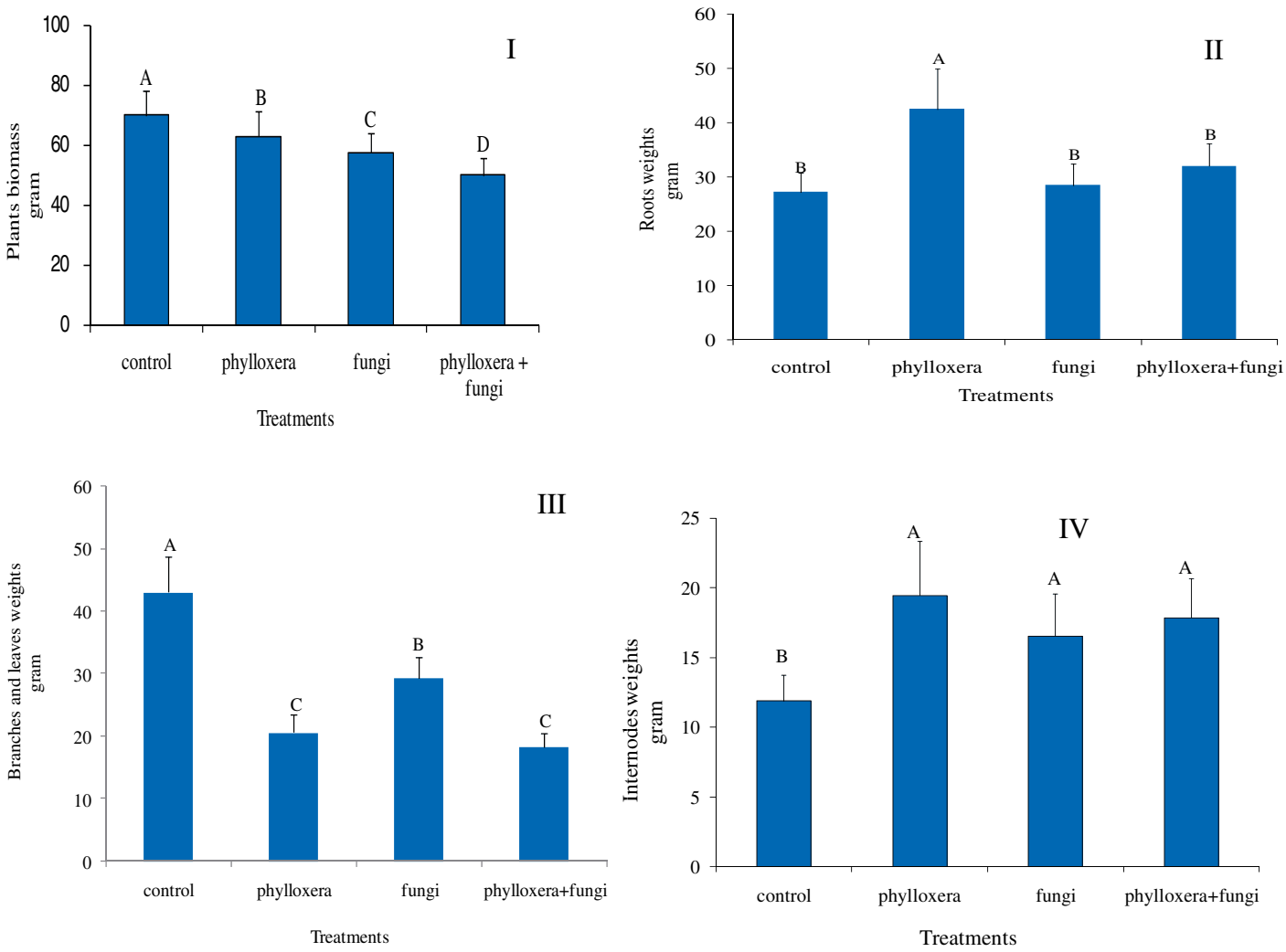

Fig. 1 - Effect of different treatments on plant biomass (I), root (II), branches + leaves weights (III) and internode weights (IV).

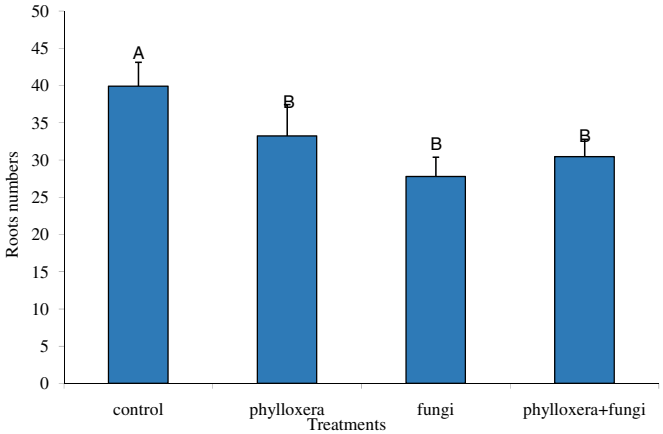

Fig. 2 - Effects presents of phylloxera and fungi on roots numbers

SY7 to spread throughout the entire plant 100%. However, the other two fungi (*F. culmorum SY24* and *F. equiseti SY3*) species may not possess this mechanism to spread in the phloem parenchyma and eventually they are not able to infect the vine despite the presence of phylloxera which may be attributed either to the lack of proper growth condition in vine or to the presence of immune response in the plant which prevents the growing of the later fungi species (Omer *et al*., 1999; Fossen, 2002).

To confirm the effect of *F. solani SY7* and phylloxera on vines, we established an experiment to evaluate five parameters (the weights of plant biomass, root internodes, roots, branches and leaves and root numbers in presence or absence of phylloxera compared to control plants). From these biological parameters, we can estimate the relationship between *F. solani SY7* and phylloxera. Omer *et al*.

Table 3 - Effects of *F. solani SY7* infection on numbers of nymphs and tuberosities

Treatments	Nymphs number (SE)	Tuberosities number (SE)
Phylloxera	25 ± 1 a	4.9 ± 0.9 a
F. solani SY7	0	0
Phylloxera + *F. solani* SY7	12.9 ± 0.9 b	3.4 ± 0.5 b
Control	0	0

Data represents nymphs number mean in tested roots of 16 plants in each treatment. Data were subjected to ANOVA analysis and the differences between means were tested for significance using Tukey HSD test. Means followed by different letters (columns) are significantly different at P <0.05.

(1995) demonstrated that greenhouse experiments with effects of fungal infection on grapevine vigor after pruning at week 13 showed that damage was significantly greater in vines infested by phylloxera, *F. solani* and *Pythium ultimum* than the damage in vines infested with phylloxera alone. Total biomass was reduced by 16% in vines infested with phylloxera and by 24% in vines infested by phylloxera and *F. solani* (Omer *et al*., 1995). In our study it was found that the total biomass was reduced by 9% in vines infested with phylloxera and by 29% in vines infested with phylloxera and *F. solani SY7*, compared to the controls. The differences between *F. solani SY7* and *F. solani* (Omer *et al*., 1995) may be attributed to the disparity of aggressiveness of the isolates. Therefore, the infection with phylloxera and fungi together caused a synergetic effect where the plant biomass tend to decrease in presence of phylollxera or fungi. This can be attributed to the damage in the vine roots caused by either phylloxera or fungi which cause dysfunction of the root as they should be in the normal conditions.

As both phylloxera and fungi affected the roots directly therefore, more work focused on the roots. The root weight and internodes increased significantly by 36 and 63% comparing to the control, in the presence of phylloxera and fungi respectively. This increase is normal in the presence of phylloxera due to the tuberosities and nodosities formed by the phylloxera. However, fungi alone did not cause changes in the root weight (Omer *et al*., 1995).

Our results demonstrated that the significantly growth of infested vines with phylloxera alone or with a combination of phylloxera and *F. solani SY7* may attribute to the rapid growth observed after potting. Moreover phylloxera may require more time necessary to establish feeding sites (Omer *et al*., 1995).

Additionally, a reduction of 50%, 30% respectively was observed in branch and leave weight and in the number of roots in the presence of phylloxera or both phylloxera and fungi compared to control, is attributed to roots death.Therefore, *F. solani SY7* infection can spread radially causing necrosis in the parenchyma and phloem. Ultimately the infection kills the roots and eventually the plant (Omer *et al*., 1999). Similarly Fossen (2002) found that the virulence isolates in present of *V. vinifera* on vine roots led to proportion of the root circumference that became necrotic in a 5-week period. Some isolates were highly virulent, causing up to 80% necrosis while other isolates were negligibly virulent (Fossen, 2002).

It has been shown in other plant-herbivore-pathogen systems that co-occurrence can, through direct interaction or changes in host's susceptibility, affect the performance of the pest or the pathogen (Karban *et al*., 1987; Hatcher., 1995). Omer *et al*. (2002) reported that the ability of grape phylloxera to exploit grape roots increased in the absence of fungal pathogens and indicated that phylloxera on infected roots developed more slowly, and had substantially reduced survival and reproduction rates. Therefore our results revealed the presence of fungi reduced the ability of phyloxera to form tuberosities by 31%. Our data also demonstrated that the total root weight decreased in presence of fungi with the Phylloxera-infested vine roots which were also reflected by a decrease in the number of nymphs.

Therefore our assays demonstrated that phylloxera on *F. solani SY7* infected roots were developed more slowly, since the nymphs and tuberosities were significantly decreased by 49% and 31% respectively. The reproduction and feeding activities of phylloxera were significantly decreased in the presence of fungal infection, consequently this result is in agreement with Omer *et al*.(2000). Furthermore the ability of *F. solani SY7* to spread within the plant parts increased when grapevine roots were infested with phylloxera grape.

These results provide interesting piece of information about the relationship between *F. solani SY7* pathogens and phylloxera. However these results are to be proved in the field. The present study provides preliminary information that could help in application of integrating pest management (IPM) program against grape phylloxera.

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Growth of tall fescue (*Festuca arundinacea* Schreb.) seedlings sown in soil mixed with nitrogen and natural zeolite

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Key words: Clipping, soil mixture, vegetative variables.

Abstract: To determine the interaction of nitrogen and natural zeolite in culture medium on the vegetative growth of tall fescue (*Festuca arundinacea* Schreb.) 'Starlet' a greenhouse experiment was conducted. A complete randomized design with factorial arrangements including two factors (nitrogen and zeolite) was employed for each treatment with four replications. Treatments of nitrogen were 0, 0.06 and 0.12 g kg^{-1} in the soil mixture and treatments of zeolite were 0, 10, 20 and 30 g kg^{-1} in the soil mixture. Application of zeolite and nitrogen had different effects on seedling height, fresh and dry weights of clippings before first, second and third mowings, chlorophyll and nitrogen content of clippings, and dry weights of roots. Adding zeolite at the rate of 30 g kg^{-1} and nitrogen at the rate of 0.12 g kg^{-1} to culture medium significantly increased the height of turf seedlings. It is concluded that zeolite could absorb and slowly release nitrogen to the culture medium.

1. Introduction

The genus *Festuca* includes more than 360 species that differ widely in appearance. Less than ten species are used as turf, all in cool climates. *Festuca arundinacea* Schreb., tall fescue, is a deep-rooted, cool-season perennial grass. It shows vigorous growth in spring and autumn, and its extensive root system helps it to withstand drought conditions. The species is adapted to a wide range of soil and climatic conditions, but performs best where winter is rather mild. Its requirement for relatively high mowing times limits its use as lawns in parks, golf course roughs and other areas mowed at 40 mm or more (Wiecko, 2006).

Zeolites are crystalline, hydrated alumino silicates of alkali and earth metals that possess infinite, three-dimensional crystal structures. They are further characterized by an ability to lose and gain water reversibly and exchange some of their constituent elements without major changes in structure. Nearly 50 natural species of zeoiltes have been recognized and more than 100 species without natural counterparts have been synthesized in the laboratory (Breck, 1974; Meier and Olsen, 1987; Mumpton, 1999).The great effectiveness of zeolites as natural sources of trace elements supplementing NPK and their high adsorption ability have been reported (Kolyagin and Kucherenko, 2003). Therefore, natural zeolites, due to their structure and properties (inert and non-toxic), can be used as slow-release carriers of fertilizers (Ramesh *et al*., 2011).

A 10% addition of clinoptilolite to sand used in the construction of golf-course greens substantially reduced NO_3^-leaching and increased fertilizer-N uptake by creeping bent-grass without disturbing the drainage, compaction, or playability of greens (Ferguson *et al*., 1986; Nus and Brauen, 1991; Hung, 1992). The addition of NH_4^+ exchanged clinoptilolite in greenhouse experiments resulted in 59 and 53% increase in root weight of radishes in medium with light clay soil (Lewis *et al*., 1984).

The water efficiency of surface irrigation systems in Iran is low and nitrogenous fertilizers should be added to soil at a higher level. Therefore leaching of nitrogen fertilizers occurs resulting in underground water pollution particularly in soils with light texture. Clinoptilolite, a naturally occurring zeolite with high exchange capacity, may be used to absorb ammonium and retard excess leaching of nitrate. These facts dictate improving water and fertilization management to decrease the pollution of underground water resources (Abdi *et al*., 2004).

The object of the present study was to investigate the effects on tall fescue growth of applying different amounts of natural zeolite and nitrogen to growth medium.

2. Materials and Methods

A greenhouse pot trial was conducted in 2009 at the Faculty of Agriculture, Shiraz University, Shiraz, Iran, at Badjgah, 1810 m above mean sea level, 29° 36´N and 52°

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32´E on tall fescue (*Festuca arundinacea* Schreb.) 'Starlet' to evaluate N uptake from soil mixture with different amounts of zeolite. The soil samples were collected for soil nutrition analysis before the seeds were planted (Table 1).

Seeds of tall fescue cultivar of Starlet were planted in 3 kg pots with soil mixture (1:1 v/v field soil and sand). The soil mixture of pots contained 0, 10, 20 and 30 g kg^{-1} zeolite and 0, 0.06 and 0.12 g kg^{-1} Nitrogen (in the form of urea) in different treatments (4 levels of zeolite × 3 levels of Nitrogen × 4 replication= 48 pots). Plants were maintained in a greenhouse under natural light (>850 µ mol m^{-2} s^{-1}) with diurnal temperature 26±3°C and nocturnal temperature 20±3°C, and RH of 56±4%. Turf grasses were irrigated every four days in spring and every two days in summer. One month after seed germination the first mowing was carried out; second and third mowings occurred 2 and 3 months after germination of seeds, respectively. Seedling height before first, second and third mowings, fresh and dry clipping weights after each mowing, chlorophyll and Nitrogen content of clipping, and dry weights of roots were measured. Seedling heights were measured. Chlorophyll content was determined by spectrophotometric method (Saini and Buvalda, 1998). The total amount of nitrogen (N) was measured using the Kjeldahl digestion method. Clippings and roots were weighed for fresh weight and then oven dried at 70°C (Karl Kolb 112SL) for 48 h and weighed for dry weight.

This research was carried out in a complete randomized design with factorial arrangements including two factors (Nitrogen and Zeolite) for each treatment with four replications from April to August 2009. Data were analyzed by MSTATC software and mean values were compared using the LSD test at 5% level.

3. Results

Seedling height before first, second, and third mowings (cm)

Increasing the content of zeolite in the culture medium to 0.12 g kg^{-1}nitrogen, seedling height increased before all mowing times. The effect of zeolite alone on seedling height had shown increase, this increase was significant at 30 g kg^{-1} zeolite in soil mixture before first and second times of mowing. Increasing the nitrogen concentration in the soil mixture had no regular and significant effect on seedling height before the three mowing times when compared to untreated control (Table 2).

Fresh and dry clipping weights after first, second, and third mowings (g)

As indicated in Figures 1, 2, and 3, an increase in zeolite and nitrogen together in the soil mixture let to greater fresh

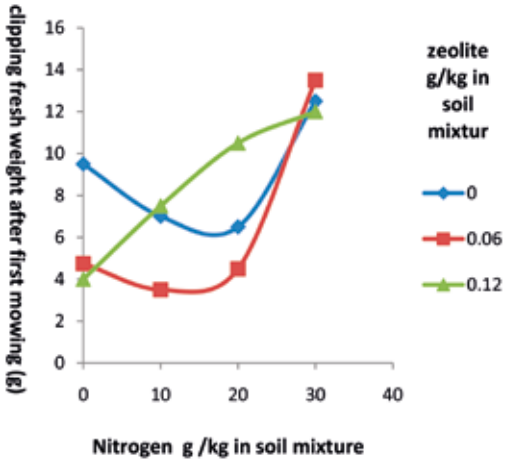

Fig. 1 - Effect of nitrogen and zeolite on clipping fresh weight after first mowing (g) of *Festuca arundinaceae* Schreb'Starlet'..

Table 1 - Some physical, chemical and nutritional characteristics of the soil used in the study

CEC	pH	N %	P (mg kg^{-1})	K (mg kg^{-1})	OC (%)	S.P.	Clay (%)	Silt (%)	Sand (%)
1.3	7.53	0.26	27.5	400	2.72	26	12	34	54

Table 2 - Effect of nitrogen and zeolite on seedling height before first, second and third mowings of *Festuca arundinaceae* Schreb 'Starlet'

	Seedling height before first mowing (cm)			Means of zeolite	Seedling height before second mowing (cm)			Means of zeolite	Seedling height before third mowing (cm)			Means of zeolite
	Zeolite g·kg^{-1} soil mixture				Zeolite g·kg^{-1} soil mixture				Zeolite g·kg^{-1} soil mixture			
	0	0.06	0.12		0	0.06	0.12		0	0.06	0.12	
Nitrogen g kg^{-1} in soil mixture												
0	20.50 ab*	18.25 abc	13.75 cd	17.50 B	17.50 bcd	18.00 bcd	13.50 d	16.33 B	15.25 a	13.50 abc	11.25 bc	13.33 AB
10	22.50 ab	13.25 d	22.75 ab	19.50 AB	19.00 abc	13.50 d	18.75 abcd	17.08 B	14.25 abc	11.00 c	13.75 abc	13.00 B
20	16.50 bcd	20.0 abcd	22.00 ab	19.50 AB	16.00 cd	17.75 bd	22.00 ab	18.58 B	13.50 abc	14.00 abc	13.50 abc	13.67 AB
30	21.50 ab	23.00 ab	24.25 a	22.92 A	20.25 abc	22.00 ab	24.00 a	22.08 A	15.00 ab	15.25 a	15.50 a	15.25 A
Mean of Nitrogen	20.25 A	18.63 A	20.69 A		18.19 A	17.81 A	19.56 A		14.50 A	13.44 A	13.50 A	

* In each column and row means followed by the same letter(s) (small letters for means and capital letters for means of rows and columns) are not significantly different using LSD test at 5% level.

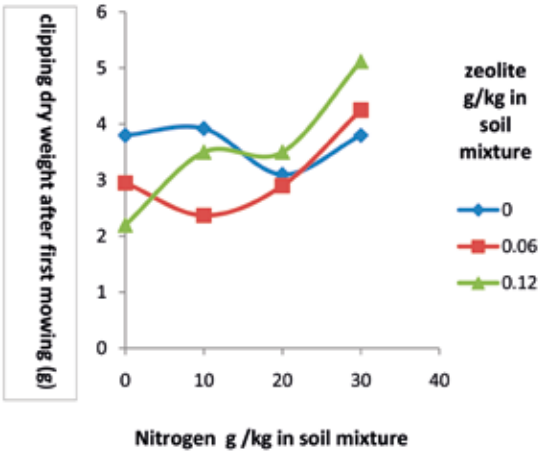

Fig. 2 - Effect of nitrogen and zeolite on clipping dry weight after first mowing (g) of *Festuca arundinaceae* Schreb'Starlet'.

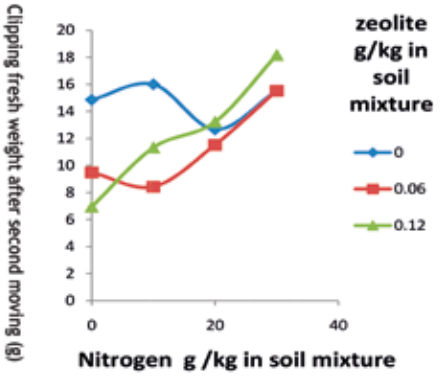

Fig. 3 - Effect of nitrogen and zeolite on clipping fresh weight after second mowing (g) of *Festuca arundinaceae* Schreb'Starlet'.

and dry weights of the clippings from the first, second, and third mowings. The means of zeolite had shown increase with increasing in zeolite content of soil mixture and this increase was significant only at 30 g kg^{-1} zeolite in soil mixture at fresh weight of clipping after all times of mowings. Also 30 g kg^{-1} zeolite in soil mixture had shown significant increase at dry weight of clipping after first and second times of mowings. The mean nitrogen content in the soil mixture had no significant effect on the fresh and dry weights of clippings for all mowing times (Figs. 1, 2, 3, 4, 5 and 6).

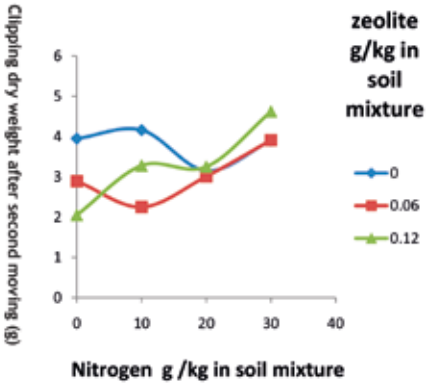

Fig. 4 - Effect of nitrogen and zeolite on clipping dry weight after second mowing (g) of *Festuca arundinaceae* Schreb'Starlet'.

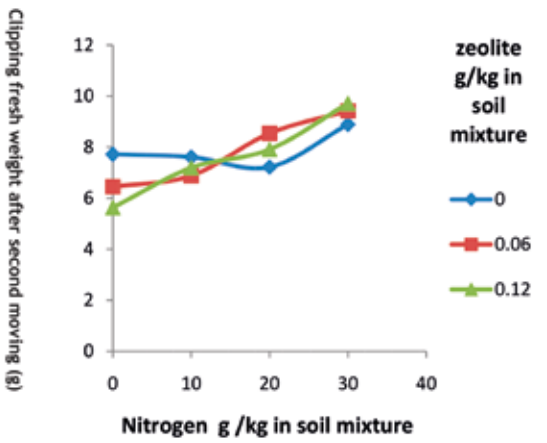

Fig. 5 - Effect of nitrogen and zeolite on clipping fresh weight after third mowing (g) of *Festuca arundinaceae* Schreb'Starlet'.

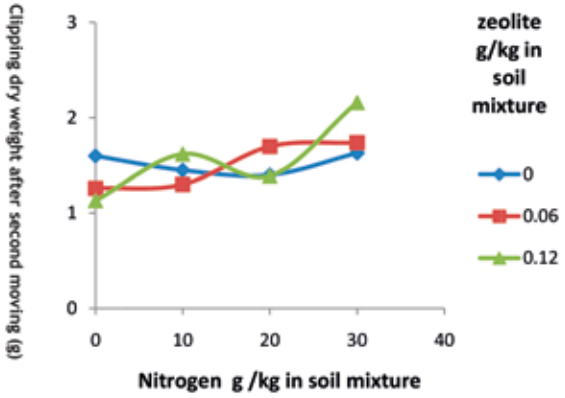

Fig. 6 - Effect of nitrogen and zeolite on clipping dry weight after third mowing (g) of *Festuca arundinaceae* Schreb'Starlet'

Chlorophyll and nitrogen content of clippings

With increasing nitrogen and zeolite in the soil mixture, the chlorophyll content of clippings decreased in most treatments but it was not significant when compared with control. Maximum chlorophyll content of clippings was found at 10 g/kg zeolite with 0.12 g/kg nitrogen in the soil mixture. The nitrogen content of clippings increased when zeolite and nitrogen increased in most treatments but this increase was not significant compared to control. Maximum nitrogen content of clippings was noted at 0 g/kg of zeolite and 0.12 g/kg of nitrogen in the soil mixture (Table 3).

Dry weight of roots

Results of this study indicate that by increasing the zeolite content alone in culture medium, the mean dry weight of roots (compared with control) increased but not significantly. An increase in the culture medium of nitrogen alone did not significantly effect the mean of root dry weight; with increasing nitrogen only, dry weight of roots decreased. However, adding nitrogen and zeolite together to the culture medium increased the dry weight of roots (Fig. 7).

Table 3 - Effect of nitrogen and zeolite on chlorophyll and nitrogen content of clippings of *Festuca arundinaceae* Schreb 'Starlet'

	Chlorophyll content of clipping			Means of zeolite	Nitrogen content of clipping			Means of zeolite
	Zeolite g·kg^{-1} soil mixture				Zeolite g·kg^{-1} soil mixture			
	0	0.06	0.12		0	0.06	0.12	
Nitrogen g kg^{-1} in soil mixture								
0	20.40 a*	17.47 a	19.60 a	19.16 A	01.12 ab	01.22 ab	03.36 a	01.90 A
10	13.85 a	14.78 a	20.86 a	16.50 A	01.49 ab	01.37 ab	01.45 ab	01.43 A
20	19.98 a	15.04 a	17.25 a	17.42 A	01.01 b	01.40 ab	00.925 b	01.11 A
30	14.89 a	16.55 a	13.22 a	14.89 A	01.12 ab	01.41 ab	01.79 ab	01.44 A
Means of Nitrogen	17.28 A	15.96 A	17.73 A		01.18 A	01.35 A	01.88 A	

* In each column and row means followed by the same letter(s) (small letters for means and capital letters for means of rows and columns) are not significantly different using LSD test at 5% level.

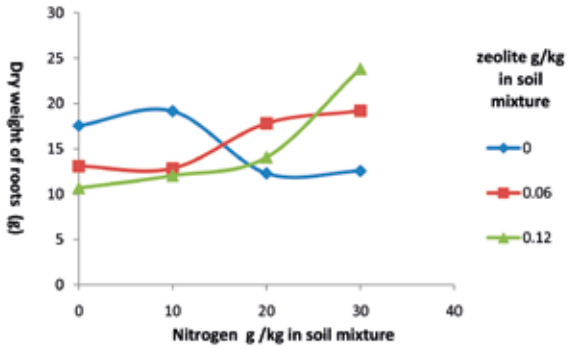

Fig. 7 - Effect of nitrogen and zeolite on clipping dry weight of roots (g) of *Festuca arundinaceae* Schreb'Starlet

4. Discussion and Conclusions

Application of zeolite and nitrogen to the culture medium increased the seedling height before all mowing times. Zeolite absorbs available N (NH_4^+) and releases it gradually to the medium for use by plants. Results of our study indicate that nitrogen alone did not increase seedling height but combined treatment with zeolite and nitrogen increased seedling height at most levels. The findings of the present study are in agreement with those of Hung and Petrovic (1995) who reported that the application of zeolite improved nitrogen efficiency in soil about 16 to 22%. Furthermore, zeolite reduced the leaching of ammonium and nitrate 86 to 99% from the soil. Zeolite, with a high cation exchange capacity (CEC), causes easy storage and release of nitrogen. Omar *et al*. (2011) reported that the application of peat soil water and zeolite with urea significantly increased dry matter, N, P, K uptake and use efficiency in maize plants compared with urea without additives. Our results are in accordance with those of Sepaskhah and Barzegar (2010) who showed that nitrogen and zeolite application resulted in higher grain protein contents in rice and nitrogen recovery efficiency. Also Aghaalikhani *et al*., (2011) reported that amending soil with zeolite, reduced nitrogen leaching, increased canola yield and nitrogen use-efficiency by avoiding nitrogen leaching and improving soil physical properties so it may be a beneficial approach to decrease chemical fertilizer application rates and develop sustainable agriculture.

In the present study, where zeolite and nitrogen were applied to the soil mixture, the chlorophyll content of clippings decreased while the nitrogen content increased. Perhaps increased nitrogen resulted in a decrease of some elements and the amount of chlorophyll. Our results are in disagreement with Abdi *et al.* (2004) and Nazari *et al.* (2007) who reported that zeolite increases the chlorophyll content of strawberry and African marigold.

Since the application of zeolite and nitrogen, compared to nitrogen application alone, increased the fresh and dry weights of clippings, it can be concluded that natural zeolite with its high exchange capacity may absorb ammonium and release it slowly. These results are in agreement with Abdi *et al.* (2004) who reported that zeolite application increased shoot dry weight in strawberry. Therefore, zeolite balances the amount of nitrogen availability for plants and retards excess leaching of nitrate. In conclusion, natural zeolite as a slow-release compound may be recommended along with nitrogen for use in growth media to improve growth of tall fescue.

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Effect of different types of mulches on 'Newton' tomato yields and fruit cracking under plastic greenhouse conditions

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Key words: Lycopersicon esculentum M., mulching, tomato cracking.

Abstract: This study was conducted at Al-Balqa` Applied University research station to investigate the effect of different types of mulches on 'Newton' tomato yields and fruit cracking incidence under greenhouse conditions. The experiment consisted of seven treatments (black plastic, tuff gravel, clear plastic, compost, crushed stone, shredded wood, and the control); a randomized complete block design with three replicates was used. Different mulch types showed significant effects on early, medium, late, and total yields/ha of the tomato fruits. Higher early and medium yields were obtained using black and clear plastic. Compost resulted with the highest total yield. Results of this study clearly showed that mulching improves total tomato yields under greenhouse conditions. In addition, larger fruits were obtained by applying mulching. Tomato cracking was also slightly affected by the mulch types used in this study.

1. Introduction

Tomato is the leading vegetable crop grown throughout the world and it is also the number one vegetable crop in Jordan. The statistics of the Ministry of Agriculture (MOA, 2012) reveal that the total tomato area cultivated is 12344.5 ha producing an average of 73.4 tons per hectare. In addition, the statistics indicate that the total number of plastic houses is extensively increasing. In Jordan, the use of various types of mulches has become a well-established practice over recent years.

It is well known that mulching helps to maintain healthy vegetable crops. The benefits of using several types of mulches have been extensively studied and recognized. However, with the global call for organic agricultural production and reduction of pesticide and fertilizer use, mulches will continue to be used.

Peet (1992) summarized the environmental, cultural, and anatomical factors that can increase the incidence of cracking as: irregular watering, high temperatures and light, fruit anatomy, excessive rapid fruit growth, genetical differences among cultivars, high differences between day and night temperatures, and high humidity. Peet and Willits (1995) concluded that growers should reduce watering tomato cultivars that are crack-prone, particularly when yielding from the upper clusters. The cuticular membrane in the outer epidermal periclinal walls of both resistant and intermediate cultivars was thicker when compared to cultivars that exhibit cracking (Matas *et al*., 2004). Cracking is mainly caused by extreme changes in fruit growth rate caused by moisture fluctuations (Swiader *et al*., 1992). Thus, the growth rate is affected by mulching material by means of manipulating the microclimate (Bender *et al*., 2008; Abubaker, 2013). Differences in the microclimate, depending on the mulch material, will influence the growth rate and hence could affect fruit cracking incidence. Therefore, the objective of this study was to investigate the effect of different types of mulches on 'Newton' tomato yield and fruit cracking incidence under plastic house conditions.

2. Materials and Methods

The research was conducted at Al-Balqa' Applied University Research Station during the 2011/2012 growing season under a green plastic house (25 m long, 9 m wide, and with a height of 2.7 m) covered with a single glaze of 200 micron clear polyethylene film. During the summer season 2011, soil solarization was applied against soil borne pests. Soil was then disked and prepared for laying the types of mulches used in the study. Seven types of mulches were used: black plastic, tuff gravel (thickness of covering 6-7 cm), clear plastic, compost (thickness of covering 6-7 cm), crushed stone (thickness of covering 6-7 cm), shredded wood (thickness of covering 6-7 cm), and the control (no

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mulch). These were arranged in a randomized complete block design with three replicates for each mulch type. Five- to six-week-old seedlings of the commercial indeterminate tomato (*Lycopersicon esculentum* M. var. 'Newton'), with an average of 12 cm height, were transplanted in December 2011. Each experimental unit consisted of a 3 m row length, with a 1 m space between every two rows. Seedling distances were 30 cm between plants with ten plants per plot. The soil surface was covered with the designated type of mulch just before transplanting and it remained covered until the end of the growing season. Tomato plants were then trained to one stem by continuous removal of auxiliary shoots. Soil moisture content was monitored using a MPKIT-160 soil moisture meter. A drip irrigation system was employed to irrigate and fertilize the plants according to local commercial tomato, plastic-house grower practices. Weeds were removed by hand and pest management control practices were applied throughout the study. Growth parameters (plant height, stem diameter, total number of leaves, and dry matter) were recorded (Abubaker, 2013). Total yield of ripe fruits was determined by recording the consecutive weights of 24 hand-harvested fruits from 6 March to 10 June 2012. Total yield was subdivided into three categories: early, medium, and late yields consisting of eight harvests each. The total number of cracked fruits and the percentages were recorded for the three harvest dates. All statistical analyses were performed using SAS/STAT Version 9.2 and Analysis of Variance was conducted by the PROC GLIMMIX procedure. Means were separated following the Fisher's Protected Least Significant Difference (LSD) Test.

3. Results and Discussion

The different mulch types showed significant effects on early, medium, late, and total yields (t/ha) of the tomato fruits (Table 1). Tomato plants grown under black, clear plastic, and shredded wood mulches resulted in the highest early yields, which were significantly higher than that of the control (Table 1). However, tuff gravel, crushed stone, and the plants grown using compost resulted in higher early yields compared to the control but they were not significantly different. Swiader *et al*. (1992) mentioned that black, gray, and transparent mulches raise soil temperatures. Thus, the rate of growth for plants grown under those mulches will increase. The mean yields showed fewer differences between the various types of mulches (Table 1). Black and clear plastic resulted in higher mean yields, however they were not significantly different from that of the control. Compost also resulted in higher mean yields that were not significantly different from the control and the slow release of nutrients from the organic material early in the season may be a possible explanation. Tomato plants that were grown using shredded wood and tuff gravel as a mulches recorded the lowest mean yields: 56.1 and 57.4 t/ha, respectively (Table 1). However, these yields were not significantly different compared to the control. Late yields showed less difference among the various types of mulches (Table 1), although the highest were recorded with tuff gravel (60. 2 t/ha) and compost (59.7 t/ha). These yields were significantly different compared to the yields of plants growing without mulch. Shredded wood also resulted in higher late yields that were significantly compared to the control. In addition, the control yields (48.1 t/ha) showed higher late yields but they were not significantly different compared to the clear plastic (46.4 t/ha), black plastic (43.5 t/ha), and crushed stone (43.3 t/ha). The highest overall yields were obtained using compost (Table 1). The descending order from highest to lowest total yields was found to be: compost, black plastic, clear plastic, tuff gravel, shredded wood, control, and finally crushed stones. The total yields of plants grown under compost, black plastic, clear plastic, and tuff gravel were significantly different compared to the control but were not significantly different compared to the shredded wood mulch. Crushed stone mulch gave the lowest total yields (144.2 t/ha), which were not significantly different compared to the control and shredded wood much. The soil temperature-increasing effect of black mulch and the late season release of organic material from the compost explain the ability of such mulches to give superior production compared to the other types. Our results coincide with Kayum *et al*. (2008) and Bay (2011). Both researchers showed that

Table 1 - Yield (early, medium, late, and total) and average fruit weight of greenhouse tomato 'Newton' under different types of mulches

Mulch type	Early yield (t/ha)	Medium yield (t/ha)	Late yield (t/ha)	Total yield (t/ha)	Fruit weight (g)
Black plastic	46.5 a	70.1 a	43.5 b	160.1 a	173.4 ab
Tuff gravel	38.7 bc	57.4 b	60.2 a	156.3 a	169.5 abc
Clear plastic	45.2 a	66.3 a	46.4 b	157.9 a	174.7 a
Compost	36.7 c	66.0 a	59.7 a	162.4 a	175.1 a
Crushed stone	37.7 bc	62.2 ab	43.3 b	144.2 b	150.5 d
Shredded wood	43.3 ab	56.1 b	56.2 a	155.6 ab	160.8 bcd
Control	33.2 c	63.3 ab	48.1 b	144.6 b	160.2 cd
LSD 0.05	6.29	8.11	7.45	11.48	13.15

Different letters in a column indicate significant differences at $P \leq 0.05$ according to Fisher's Protected LSD.

mulching significantly affected yield components, increased yield, and improved fruit quality.

Regarding fruit weight, the largest fruits were obtained using compost, clear and black plastic with average weights of 175.1, 174.7, and 173.4 g/fruit (Table 1). These weights were not significantly different compared to tuff gravel. The lowest fruit weights were achieved when crushed stone was used, followed by the control and shredded wood. There were no significant differences with regard to the lowest fruit weights.

Dry matter contents of leaves and stems were significantly affected by mulch types (Table 2). Tomato dry matter was highest when grown using the compost as a mulching material (19.4%); differences with crushed stone (18.8%) and black plastic (18.3%) were not significant. Abubaker (2013) attributed similar findings to the higher amounts of available minerals released from the compost which also manifested favorable effects on available water content during the growing season, directly affecting vegetative growth.

Table 2 - Dry matter percentages of 'Newton' tomato leaves and stems grown under different types of mulches

Mulch type	Dry matter of leaves and stems (%)
Black plastic	18.3 ab
Tuff gravel	17.8 b
Clear plastic	17.6 b
Compost	19.4 a
Crushed stone	18.8 ab
Shredded wood	17.7 b
Control	17.6 b
LSD 0.05	1.30

Different letters in a column indicate significant differences at $P \leq 0.05$ according to Fisher's Protected LSD.

Regarding fruit cracking, average values are presented in Figure 1 and the average percentage of cracked fruits are presented in Figure 2. The results clearly indicate no significant differences between the seven mulch types used in our study. However, the average number of cracked fruits was highest when compost was used, and it is worth mentioning again that the highest overall yields were obtained using this type of mulch. However, slight differences were observed in the average percentage of cracked fruits with regard to the total number of fruits, particularly between the clear plastic and control treatments but these differences were not significantly different. Our results are in agreement with Suwwan *et al*. (1988) who indicated that seasonal cracking was not affected by the five mulch types they studied (i.e. silver plastic, black plastic, paper, white/black plastic and black/white plastic).

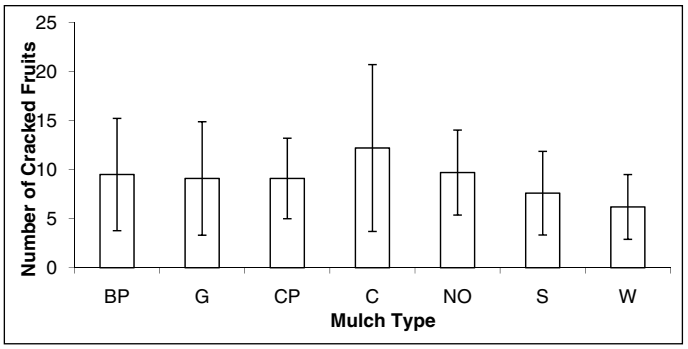

Fig. 1 - The average number of cracked `Newton` tomato fruits for the three harvest dates for each mulch type. BP= black plastic, G= gravel, CP= clear plastic, C= compost, NO= no mulch (control), S= stone, and W= wood.

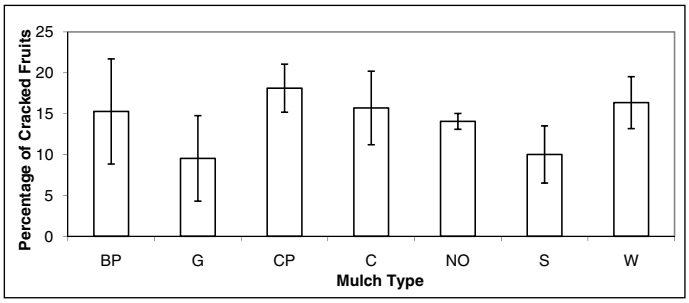

Fig. 2 - The percentage of cracked `Newton` tomato fruits for the three harvest dates for each mulch type. BP= black plastic, G= gravel, CP= clear plastic, C= compost, NO= no mulch (control), S= stone, and W= wood.

4. Conclusions

Mulching improved growth parameters and yields of 'Newton' tomato grown under plastic house conditions. The highest overall yields were obtained using compost followed by black plastic. No significant differences among types of mulches were observed regarding the number of cracked fruits. However slight, though not significant, differences were found when considering the average number of cracked fruits compared to the total number of fruits.

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Bermudagrass adaptation in the Mediterranean climate: phenotypic traits of 44 accessions

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Key words: colour, *Cynodon dactylon*, green-up, node density, quality, shoot density.

Abstract: The use of bermudagrass in the Mediterranean area is increasing for its outstanding tolerance to heat and drought, and its aggressive growth and high recuperative potential make it particularly suited to heavily worn areas and appreciated for sports turfs. However, the overall performance of a given genetic type can be affected by the adaptation to a specific environment. The objective of this research was to determine the variability of a number of phenotypic traits that can affect bermudagrass turf performance on a wide range of bermudagrass accessions grown in two locations in Italy. In May 2010, 44 accessions of bermudagrasses, grouped in "wild", "improved" "hybrid" and "dwarf" types were transplanted in the center of field plots in Pisa and Palermo. In 2011, when the turf was completely established, the following traits were determined: shoot density, horizontal stem density, node density, leaf width, colour, quality, spring green-up, and fall colour retention. Dwarf and hybrid types yielded the best aesthetic characteristics. With respect to colour retention and spring green-up, great variability was recorded within the groups. Dwarf types presented the earliest dormancy, while the hybrid types were in general the ones to green-up first in spring.

1. Introduction

Bermudagrass is still the dominant warm-season turfgrass in warm to temperate climatic regions of the world. It is well adapted to a wide range of soil types, and its drought tolerance, recuperative ability, salt tolerance, wear tolerance, aggressive stoloniferous and rhizomatous growth habit, and overall appearance make bermudagrass an ideal turfgrass in many environments (Taliaferro, 2003; Shearman, 2006).

Bermudagrass includes several taxa of the genus *Cynodon* (L.) Rich. but the two species that represent the genetic pool from which the present cultivars descend are *Cynodon dactylon* (L.) Pers. Var. *dactylon* and *Cynodon transvaalensis* (Burtt-Davy) also known as African bermudagrass (Taliaferro, 2003). *C. transvaalensis* is morphologically distinct from *C. dactylon* due to narrow erect pale leaves producing a fine textured turf with a yellowish-green colour (De Wet and Harlan, 1970; Taliaferro, 1992). *C. dactylon* can be found as far north as 53° N latitude and from sea level to 3000 m altitude (Taliaferro, 2003). Asexual reproduction has played a role in bermudagrass enhancement as well. Remarkable breeding progress has been obtained from inter-specific hybridization and mutation breeding. The inter-specific hybridization of *C. dactylon* and *C. transvaalensis* has been extensively used to obtain sterile cultivars for which clonal propagation is necessary due to a lack of viable seeds. Among hybrid genotypes, a number of cultivars have been selected for plant size and morphology in response to the lower cutting height adopted over the years on golf greens. The more recently released "ultradwarf" cultivars have become routinely adopted thanks to an improved density, a slower vertical leaf extension and an increased dominance of stoloniferous growth relative to rhizomes at low mowing heights (Beard and Sifers, 1996).

Parameters used to evaluate *Cynodon* turf typically include turfgrass quality, colour, percent spring green-up, establishment rates, leaf texture, and density. As the turfgrass industry moves towards more sustainable management practices, the types of parameters potentially related to better stress tolerance are increasingly important (Baldwin and Liu, 2013).

One of the most important parameters is cold tolerance and rapidity of recovery from winter dormancy in the spring (Anderson *et al*., 2007; Patton *et al*., 2008). Low-temperature tolerance depends on a combination of sev-

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eral factors, including environmental conditions, cultural practices, and especially genetic factors (Blum, 1988; Anderson and Taliaferro, 2002). Bermudagrass survives the dormancy period using its reserves of nonstructural carbohydrates and nitrogen compounds accumulated during the previous growing season in storage organs such as stolons and rhizomes (Macolino *et al*., 2010; Volterrani *et al*., 2012; Giolo *et al*., 2013; Pompeiano *et al*., 2013).

In the last two decades several southern European universities have developed research programs to study warm-season turfgrass species, including bermudagrass (Volterrani *et al*., 2008; Lulli *et al*., 2011; Lulli *et al*., 2012; Nikolopoulou *et al*., 2012; Agati *et al*., 2013; Gómez de Barreda *et al*., 2013), and in particular their adaptability to the Mediterranean environment (Volterrani and Magni, 2004).

The aim of our research was to determine the variability of a number of phenotypic traits and aesthetical characteristics that can affect bermudagrass turf performance in a wide range of bermudagrass accessions grown in two locations in Italy. This information can provide further insight into bermudagrass adaptability in the Mediterranean climate.

2. Materials and Methods

Plant material

With the objective of expanding morphological diversity of the plant material, 44 accessions of bermudagrass [*Cynodon* (L.) Rich.], representative of both wild populations and cultivars, were included in the present study.

Group one included 13 entries that were called "wild types", naturally occurring populations of *C. dactylon* (L.) Pers. collected from contrasting environments supposed to generate a selective pressure. Collection sites were located in Italy (CeRTES-1= warm temperate, salt affected soil; CeRTES-2= warm temperate, fertile soil; CeRTES-3= warm temperate, polluted soil; CeRTES-13= warm temperate, fertile soil), France (CeRTES-4= cool humid, fertile soil), Greece (CeRTES-5, -6 and -7= warm temperate, salt affected soils), Croatia (CeRTES-8= warm temperate, salt affected soil), Argentina (CeRTES-9= warm temperate, salt affected pastureland), United Arab Emirates (CeRTES-10 and -11= warm arid, desert sand), and Maldives (CeRTES-12= tropical humid, salt affected soil).

Group two included 13 entries that were called "improved types". These were experimental or commercial vegetative and seeded improved *C. dactylon* cultivars.

Group 3 included 11 entries called "hybrid types", commercial or experimental inter-specific hybrids (*Cynodon dactylon x transvaalensis* Burtt.-Davy) of which those labelled Tif- were kindly provided by Dr. W. Hanna (University of Georgia, USA).

Group four included seven entries called "dwarf types", commercial interspecific dwarf and ultradwarf hybrid cultivars and two *Cynodon transvaalensis* Burtt.-Davy accessions, one a commercial cultivar (Uganda) and the other (Roma) a line of African bermudagrass that was collected in a turf nursery in Rome (Italy) where the species was first introduced presumably as a weed. African bermudagrasses were included in the "dwarf types" due to their similarity in leaf texture, density and growth habit with the well-known hybrid dwarf bermudagrasses.

2010

Turf establishment

On 15 April 2010 at the University of Pisa, Italy, all the accessions were propagated in the greenhouse (24±5°C) in peat-filled honeycomb seed trays (7 cm^2 area and 25 cm^3 volume each cell). Vegetatively propagated genotypes were planted as single stolon and seeded cultivars were seeded as single seed.

On 13 May 2010 plants in the greenhouse were fertilized (30 kg ha^{-1} N, 10 kg ha^{-1} P, and 10 kg ha^{-1} K) using a soluble fertilizer (Grow More Inc., Gardena, CA, USA).

On 31 May 2010, plants were mown to 5 cm and transplanted into field plots in two locations in Italy: the research station of the University of Pisa (43°40'N, 10°19'E, 6 m a.s.l.) and the research station of the University of Palermo (38°06'N, 13°20'E, 50 m a.s.l.).

Experimental plots were 1.5 by 1.5 m with 0.5 m bare soil pathways arranged in a randomized complete-block design with four replications. One plant of bermudagrass was transplanted in the centre of each plot. Soil type at Pisa was silt-loam (28% sand, 55% silt and 17% clay) with a pH of 7.8 and 18 g kg^{-1} organic matter while at Palermo soil type was sandy clay loam (54% sand, 23% silt and 23% clay) with a pH of 7.6 and 14 g kg^{-1} organic matter. Irrigation was applied as needed to encourage establishment. Plots received 50 kg ha^{-1} N, 10 kg ha^{-1} P, and 40 kg ha^{-1} K per month from June to September 2010. In order to minimize weed competition, from two years before establishment, the experimental areas were treated twice a year with glyphosate [N-(phosphonomethyl) glycine] at 2.88 kg ha^{-1} a.i. The day before planting, oxadiazon [5-tert-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2(3H)-one] was applied at 3.36 kg ha^{-1} a.i. Plots were not mowed during the year of establishment to avoid genotype x mowing interaction. Weeds occurring during the trial period were manually removed, even if it is not a standard cultivation technique, as the accessions could be differently injured by chemical removal. In the trial, no pesticides were applied. Encroachment of stolons into adjacent plots was avoided by using toothpicks that redirected the growing tip back toward the plot centre.

2011

In 2011, in the first and second weeks of March at Palermo and Pisa, respectively, scalping was carried out. From the end of April (the end of green-up) to October 2011, the turf was mowed weekly with a reel mower (John Deere 20SR7) at a mowing height of 2.5 cm. The irrigation program was adjusted according to soil temperature and evapotranspiration rate, with supplement irrigations applied as needed to prevent visual wilt of the turf (Croce *et al*., 2004). Plots received 50 kg ha^{-1} N, 20 kg ha^{-1} P, and 40 kg ha^{-1} K per month from May to August 2011.

Weeds were manually removed inside the plots during the trial period as the accessions could be differently injured by chemical treatments. In the trial, no pesticides were applied. To avoid the encroachment of stolons into adjacent plots, the corridors were treated with glyphosate at 2.88 kg ha^{-1} a.i. every other week. No turf cultivation, or verticutting or phytosanitary treatment was practiced on the plots.

Monthly mean maximum and minimum temperatures recorded at the two trial sites are reported in Table 1. There were 13 days in Pisa with air temperatures below 0°C from November 2010 to March 2011; zero days were recorded in Palermo for the same period.

Table 1 - Monthly mean air temperatures (°C) during the trial period (2011) at Pisa and Palermo

Month	Air temperature (°C)			
	Pisa		Palermo	
	Mean maximum	Mean minimum	Mean maximum	Mean minimum
January	10.6	3.5	14.7	9.3
February	11.8	3.3	13.9	9.3
March	13.9	5.2	16.2	10.4
April	19.2	9.0	19.6	13.1
May	23.8	11.8	21.4	15.9
June	26.2	17.0	25.2	20.2
July	27.6	18.3	28.6	22.9
August	30.2	18.5	29.0	23.0
September	26.7	16.0	26.9	21.0
October	21.8	10.5	22.6	16.5
November	17.5	6.6	20.2	14.5
December	13.3	4.7	16.9	12.6

Assessments

Spring green-up (15 March-15 May 2011) and fall colour retention (15 November 2011-15 January 2012) were estimated and expressed as percentage of green ground cover. In the second week of October at both testing sites a 50 cm^2 core sample per plot was collected and the following parameters determined: leaf width (20 fully expanded leaves per plot measured with a precision Vernier caliper and data reported in millimeters), shoot density (direct counting with data reported as shoot no cm^{-2}), horizontal stem density (stolons and rhizomes collected after soil washing measured with a ruler and data reported as cm cm^{-2}) and node density (nodes of stolons and rhizomes collected from the core samples counted and reported as no cm^{-2}) (Roche and Loch, 2005; Volterrani *et al*., 2008; Volterrani *et al*., 2010; Pompeiano *et al*., 2012).

At the Pisa location two additional parameters were determined: at 30-day intervals throughout the growing season (May-October) colour, with a rating scale of 1=light green and 9=dark green, and quality with a rating scale of 9 = best and 1 = poorest (Morris and Shearman, 2007; Patton *et al*., 2009) were estimated.

Data were subjected to analysis of variance using CoStat software (Monterey, CA, USA). To test the effects of location, accession and their interaction, a factorial combination was used. Significantly different means were separated using Fisher's Least Significant Difference (LSD) at the t-probability level of 0.05.

3. Results

The interaction of treatments was not statistically significant for any of the parameters recorded in Pisa or Palermo. For all the parameters, the location and accession mean effects were statistically significant. Location effect is reported as average across accessions and accession effect is reported as average across locations.

Location mean effect

For the location effect, shoot density and node density were on average higher in Pisa (3.1 shoots cm^{-2} and 2.4 nodes cm^{-2} respectively) compared to Palermo (0.8 and 1.0) (Table 2). Also horizontal stem density was higher in Pisa (4.2 cm cm^{-2}) while leaf width recorded in Pisa was on average less (1.4 mm) compared to Palermo (2.4 mm).

Spring green-up evaluations as average across locations showed green cover percentages (April 14) higher in Palermo (81%) with respect to Pisa (60%), while fall colour retention (December 17) showed higher green cover percentages in Palermo (77%).

Accession mean effect

Shoot density

All wild type entries, with the exception of CeRTES 12 (2.4 shoot cm^{-2}) which produced a density comparable to hybrid and improved types, had a similar shoot den-

Table 2 - Bermudagrass [*Cynodon* (L.) Rich.] shoot density, horizontal stem density, node density, leaf width. Location effect averaged across accessions

	Shoot density (n° cm^{-2})	Horizontal stem density (cm cm^{-2})	Node density (n° cm^{-2})	Leaf width (mm)	Spring green-up (%)	Fall colour retention (%)
Pisa	3.1	4.2	2.4	1.4	60	33
Palermo	0.8	1.9	1.0	2.4	81	77

Means are significantly different at the 0.05 level of probability as determined by Fisher's protected LSD.

sity with values ranging from 0.5 to 1.0 shoots cm^{-2} (Table 3). The most dense improved type was Wintergreen (2.3 shoots cm^{-2}), hybrid type values ranged from 1.7 shoots cm^{-2} (Patriot) to 3.8 shoots cm^{-2} (Tif 00-1), while the most dense dwarf type was Miniverde with 5.1 shoots cm^{-2}.

Horizontal stem density

The highest value was recorded for Miniverde with 5.5 cm cm^{-2} while CeRTES 12 had a slightly lower value (5.1 cm cm^{-2}) (Table 3). The variability within the different groups was high for this parameter with values ranging from 1.5 to 5.1 cm cm^{-2} (respectively for Certes 1 and Certes 3 versus CeRTES 12) for the wild types, from 2.1 to 4.9 cm cm^{-2} (respectively for Scotts R6LA and Yukon versus Bull's Eye) for the improved types, from 2.7 to 4.3 cm cm^{-2} (for Tif 00-18 compared to Santa Ana and Tif 00-1) for the hybrid types, and from 1.6 to 5.1 cm cm^{-2} (respectively for Tifdwarf and Miniverde) for the dwarf types.

Node density

CeRTES 3 and Miniverde were the entries with the lowest and the highest node density with 0.6 and 5.5 nodes cm^{-2}, respectively (Table 3). The variability within the groups was high for this parameter, with the exception of the hybrid types that had values ranging from 1.6 to 2.5 nodes cm^{-2} (respectively for Tif 00-18 and Santa Ana).

Leaf width

Coarser leaves were found in the wild types with values ranging from 2.1 mm (CeRTES 10) to 3.0 mm (CeRTES 5, 6, 7) with CeRTES 12 (1.1 mm) the exception (Table 3). Improved *Cd* types had a leaf width ranging from 1.6 mm (Wintergreen and Yukon) to 2.2 mm (Scotts R6LA and Sovereign). For hybrid types values ranged from 1.3 mm (Tif 00-10) to 1.9 mm (Tif 00-27), while for dwarf types values ranged from 1.0 mm (Roma) to 1.5 mm (Uganda).

Spring green-up

Green cover percentage evaluated in mid-April showed accessions scoring values above 80% and not statistically different from each other in each group (Table 4). In more detail, the accession with the best score was CeRTES 5 (93%); the lowest score was found in Sovereign (24%).

Fall colour retention

Green colour retention evaluated in mid-December showed a great variability within the groups (Table 4). The highest value was recorded for Tif 00-2, with the lowest for three dwarf types, Miniverde, Tifdwarf and Tifeagle (11%).

Colour

In Pisa, the highest and lowest values, Barazur (score 8.4) and Riviera (score 6.0) respectively, were recorded within the improved type group. In the wild type group, the values ranged from 6.1 (CeRTES 1) to 7.4 (CeRTES 4 and 9) (Table 4). With the exception of Barazur, the highest values were recorded within the hybrid type group with values ranging from 7.1 (Tifsport) to 8.2 (Patriot), while

Table 3 - Bermudagrass [*Cynodon* (L.) Rich.] accessions. Shoot density, horizontal stem density, node density and leaf width. Accession effect averaged across locations

Accessions	Shoot density (n° cm^{-2})	Horizontal stem density (cm cm^{-2})	Node density (n° cm^{-2})	Leaf width (mm)
Wild types (Cd)				
CeRTES-1	0.8	1.5	0.7	2.8
CeRTES-2	0.7	1.7	0.8	2.8
CeRTES-3	0.9	1.5	0.6	2.8
CeRTES-4	0.8	2.9	1.8	2.5
CeRTES-5	0.9	1.9	0.9	3.0
CeRTES-6	1.0	3.1	1.7	3.0
CeRTES-7	0.6	2.1	0.9	3.0
CeRTES-8	0.5	1.8	0.8	2.9
CeRTES-9	0.9	2.3	0.9	2.8
CeRTES-10	0.9	2.1	0.9	2.1
CeRTES-11	1.0	2.5	1.1	2.7
CeRTES-12	2.4	5.1	3.0	1.1
CeRTES-13	0.7	1.9	1.1	2.8
Improved types (Cd)				
Argentina	1.0	4.0	1.8	2.1
Barazur	2.0	3.1	2.1	1.7
Bull's Eye	1.9	4.9	2.4	1.9
Celebration	1.3	2.5	1.3	1.9
Grand Prix	2.2	2.9	1.6	1.8
Princess 77	2.1	3.6	1.9	1.8
Riviera	1.1	2.3	0.9	1.9
Scotts R6LA	1.3	2.1	1.0	2.2
Sovereign	0.9	3.3	1.4	2.2
SR 9554	1.3	2.2	0.7	2.0
Veracruz	1.3	3.4	1.6	1.8
Wintergreen	2.3	2.9	1.5	1.6
Yukon	1.2	2.1	1.1	1.6
Hybrid types (Cdxt)				
Patriot	1.7	3.9	2.0	1.8
Santa Ana	3.1	4.3	2.5	1.4
Tifsport	2.3	3.4	1.7	1.8
Tifway	2.6	3.8	2.0	1.4
Tif 00-1	3.8	4.3	2.3	1.5
Tif 00-2	3.2	3.3	2.1	1.5
Tif 00-7	3.2	3.2	2.2	1.5
Tif 00-10	3.5	3.7	2.0	1.3
Tif 00-18	2.7	2.7	1.6	1.4
Tif 00-24	2.0	3.7	1.9	1.4
Tif 00-27	2.3	3.6	2.1	1.9
Dwarf types (Cdxt/Ct)				
Champion	2.2	4.0	2.0	1.2
Miniverde	5.1	5.5	5.5	1.3
Tifdwarf	2.6	1.6	1.0	1.3
Tifeagle	3.1	4.4	4.2	1.1
Tifgreen	2.7	1.9	1.5	1.2
Roma	4.1	3.7	2.2	1.0
Uganda	3.0	4.7	2.6	1.5
LSD 0.05	0.7	1.6	1.2	0.3

Table 4 - Bermudagrass [*Cynodon* (L.) Rich.] accessions. Spring green-up (April 14 2011) and fall colour retention (percentage of green colour) (17 December 2011). Accession effect averaged across locations. Colour (visual estimation based on a 1-9 scale) and quality (visual estimation based on a 1-9 scale) refer only to the Pisa location (mean values May-October 2011)

Accessions	Spring green-up (%)	Fall colour retention (%)	Colour (1-9)	Quality (1-9)
Wild types (Cd)				
CeRTES-1	55	16	6.1	4.3
CeRTES-2	75	32	7.0	5.7
CeRTES-3	66	38	6.3	5.8
CeRTES-4	65	38	7.4	6.8
CeRTES-5	93	76	6.4	6.0
CeRTES-6	73	49	7.2	6.4
CeRTES-7	74	37	7.1	6.2
CeRTES-8	59	33	6.6	6.2
CeRTES-9	85	63	7.4	6.7
CeRTES-10	51	53	6.7	5.9
CeRTES-11	72	68	6.2	5.6
CeRTES-12	79	54	6.8	7.0
CeRTES-13	60	41	6.7	5.9
Improved types (Cd)				
Argentina	66	45	6.4	6.1
Barazur	55	43	8.4	7.7
Bull's Eye	76	59	7.9	7.7
Celebration	49	59	7.5	6.4
Grand Prix	77	78	6.8	6.9
Princess 77	72	73	6.9	6.9
Riviera	57	50	6.0	5.8
Scotts R6LA	73	57	6.9	6.4
Sovereign	24	56	6.4	6.3
SR 9554	67	56	6.8	6.5
Veracruz	71	61	6.8	6.9
Wintergreen	83	69	7.1	6.5
Yukon	70	35	7.3	7.1
Hybrid types (Cdxt)				
Patriot	75	33	8.2	8.1
Santa Ana	77	73	7.4	7.9
Tifsport	79	68	7.1	7.2
Tifway	87	83	7.9	8.1
Tif 00-1	86	77	7.9	7.5
Tif 00-2	89	86	7.2	7.6
Tif 00-7	62	75	7.9	6.8
Tif 00-10	82	81	8.1	7.8
Tif 00-18	84	80	8.0	8.0
Tif 00-24	74	79	7.7	7.0
Tif 00-27	87	81	7.4	7.6
Dwarf types (Cdxt/Ct)				
Champion	86	76	7.7	8.0
Miniverde	53	11	7.9	8.4
Tifdwarf	63	11	7.8	7.9
Tifeagle	52	11	7.4	8.1
Tifgreen	72	31	7.0	7.9
Roma	75	56	7.4	8.0
Uganda	84	78	7.8	7.9
LSD 0.05	17	7	0.5	0.7

for the dwarf types the values ranged from 7.0 (Tifgreen) to 7.9 (Miniverde).

Quality

In Pisa, the highest quality was recorded for the dwarf type Miniverde with a score of 8.4, however no significant differences were recorded within this group (Table 4). Wild types ranged in quality from 4.3 (CeRTES 1) to 7.0 (CeRTES 12), while improved types ranged from 5.8 (Riviera) to 7.7 (Barazur and Bull's Eye); the variability of ratings within both these groups is worthy of note.

The hybrid types scored from 6.8 (Tif 00-7) to 8.1 (Patriot and Tifway).

4. Discussion and Conclusions

The study carried out on a pool of genetically and morphologically different entries belonging to the genus *Cynodon* has highlighted a wide variability of aesthetic and morphological traits.

Morphological characteristics such as shoot density, node density, and horizontal stem density highlighted the better quality of the majority of dwarf and hybrid type cultivars, with improved types showing performances similar to those of wild types; CeRTES 12 was the exception.

The genetic differences among groups are reflected more clearly with regard to leaf width, with values getting lower going from wild to dwarf types, with the exception of CeRTES 12.

Recovery from winter dormancy in the spring, expressed as spring green up, showed a great variability within and among the groups. This parameter is associated with carbohydrate reserves accumulated in storage organs as observed by Macolino *et al*. (2010). Other studies (Volterrani *et al*., 2012) focused on carbohydrates in stolons in the first year of establishment and the relationship with growth and establishment rate.

Cold tolerance, expressed as fall colour retention, highlighted the better performances of the hybrid types with the dwarf types being the first cultivars in which dormancy begins.

The parameters representing turf aesthetic quality (colour and turf quality), although they indicate a great variability within groups, showed improving mean values from wild to dwarf types and confirmed what Patton *et al*. (2009) observed concerning the differences between improved and hybrid types.

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Influence of arbuscular mycorrhizal fungi on physiology and fruit quality of Pepino (*Solanum muricatum* Ait.) in vermicompost amended medium

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Abstract: The association level of pepino (*Solanum muricatum* Ait.) with two arbuscular mycorrhizal fungi (AMF) species (*Glomus etunicatum* and *G. versiforme*) was evaluated for the first time. The first part of experiment showed 30 and 50% root colonization for the two AMF species, respectively, while the second part of study was a pot experiment under greenhouse conditions. The effects of vermicompost and root inoculation with *G. etunicatum* and *G. versiforme* on reproductive stage, yield and fruit quality of pepino were investigated. Treatments included two levels of vermicompost (0 and 20% v/v) and inoculation with the two fungi species along with a non inoculated control. Application of vermicompost increased the number of flowers, fruits and fruit weight, but decreased the number of days from plant setting to first flower and fruit set, fruit dry matter percent, fruit titratable acidity and vitamin C content. Inoculation with *G. versiforme* increased fruit dry matter percent, fruit titratable acidity and fruit vitamin C content compared with the non inoculated control (NIC) plants. Plants inoculated with *G. etunicatum* showed greater fruit weight and juice pH compared to NIC plants. AMF inoculation in vermicompost amended pots led to 14 and 10 days earlier flowering for *G. versiforme* and *G. etunicatum*, respectively compared to those not amended with vermicompost. *G. etunicatum* in vermicompost supplemented medium hastened fruit set by 5.5 days compared to those without vermicompost application. Fruit quality characteristics were affected differently for the two AMF-inoculated plants in presence of vermicompost.

1. Introduction

Pepino (*Solanum muricatum* Ait.), a little-known herbaceous subshrub Solanaceous plant, is native of the tropical and subtropical Andes in South America. It is cultivated for its edible juicy, scented and sweet fruits (Prohens *et al*., 1996) and has been introduced to different countries, becoming a specialty fruit (Ahumada and Cantwell, 1996). Today, pepino is a species of increasing economic interest and it has considerable potential for future exploitation (Prohens *et al*., 1996). The cultural techniques used in modern tomato growing have been adapted with slight modification to pepino management (Dennis *et al*., 1985).

Symbiotic associations between arbuscular mycorrhizal fungi (AMF) and plant roots in the natural environment provide a range of benefits to the host plant, however many conventional agricultural practices are detrimental to AMF (Gosling *et al*., 2006). Organic farming systems may be less detrimental to AMF, because they exclude the use of chemical fertilizers and most biocides and generally have diverse rotations.

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Organic matter influences the nutrient profile, soil structure, water holding capacity and pH, all of which directly and or indirectly influence AMF development (Bagyaraj, 1991). Addition of organic amendments to soil has been reported to enhance plant biomass, mycorrhizal infectivity, and proliferation of AM fungal hyphae in soil (Joner and Jakobsen, 1995; Dai *et al*., 2011). Vermicompost as an organic fertilizer provides some essential nutrients for supporting plant growth compared with chemical fertilizers.

To the best of our knowledge, there is no published report about the association of pepino with AMF in literature. The objectives of the present study were to determine: 1) the association level of two AMF species with pepino plant; 2) the possible beneficial effects of symbiosis on plant physiology and fruit quality; and 3) the benefits of vermicompost on AMF association with pepino.

2. Materials and Methods

Pepino (cv. Kanseola) mother plants were obtained from Mashad Ferdowsi University research station, Iran. Cuttings with an average 20 cm length and four buds were rooted in a 1:1 (v:v) peat and sand mixture for four weeks. General cultural practices were according to Lopez *et al.* (2000).

AMF inocula

AMF spores were obtained from the Department of Soil Science, Faculty of Agriculture, Shiraz University, Iran. *Glomus versiforme* was isolated from a non-contaminated area of Anguran Mine, Zanjan, Iran (Zarei *et al*., 2008) and *G. etunicatum* was provided from Tabriz University, Iran; these fungi are abundant in Iranian soils (Aliasgharzadeh *et al*., 2001; Kariman *et al*., 2005). Mycorrhizal inocula were prepared through the trap culture of maize (*Zea mays* L.) on culture medium composed of autoclaved soil/quartz-sand (<1 mm) (1:4, v/v). Simultaneously, some pots containing non autoclaved soil were kept without any spore inoculation to preserve the naturally-occurring microbial association and used for non-inoculated control (NIC) treatments. After four and a half months, at the beginning of the maize reproductive period, shoots were removed and the contents of pots (mycorrhizal roots plus soil possessing fungal spores and mycelia) were maintained in polyethylene bags at 4°C. The potential of inoculants (spore numbers of 10-12 g^{-1} substrate and root colonization of 80-85%) for spore extraction, number, and evaluation of root colonization were measured based on the method described by Zarei *et al*. (2008).

Examination of arbuscular mycorrhizal symbiosis

The association level of pepino plant with two AMF species along with a NIC was evaluated in a completely randomized design with three replications. The presence of AM propagules and the percentage of root colonization was determined after eight weeks of inoculation. The grid-line intersect method was used after cleaning washed roots in 10% KOH and staining with 0.01% fuchsin acid in lactoglycerol according to the method described by Kormanik and McGraw (1982). The AMF species colonizing roots of pepino plants were used for the main experiment.

Main experiment

The experiment was carried out in a polyethylene greenhouse using 10 L pots. The experimental design was 2×3 factorial with four replications (four plants each) in a completely randomized arrangement. The first factor consisted of control (V_0: no vermicompost) and V_1: vermicompost application at 20% (v/v) to soil. The second factor was soil inoculation with *G. etunicatum* and *G. versiforme*. Non-inoculated pots were considered as control (NIC). Four-week-old rooted cuttings were planted in pots which contained the described media for treatments. The physical and chemical properties of soil and vermicompost are presented in Table 1.

For the mycorrhizal treatments 50 g of each AMF inoculum was used as a thin layer near the roots of cuttings. NIC treatments consisted of adding 50 g of media from control maize trap culture pots (contained non-autoclaved soil with no spore inoculation) as described earlier (see section 2.1). Pots containing pepino plants were arranged in rows, 1 m apart and 0.4 m between pots. Plants were trained into three main branches. A total amount of 35 $g \cdot m^{-2}$ of organic soluble fertilizer Biomin464-sp (JH Biotech, Inc. Ventura, CA) was applied to fertigated plants during the experimental period. Irrigation frequency from transplanting to harvest varied from 2 to 4 days with 0.4 - 1 L per irrigation based on plant requirements.

Plant reproductive phase measurements

To evaluate the effects of colonization with AMF and vermicompost application on the pepino reproductive stage, the following characteristics were assayed: number of days to first flower formation, number of flowers in the first and second truss, number of days from transplanting to first fruit formation, fruit number and fruit set percentage, and fruit fresh weight. Fruit quality factors including vitamin C content at maturity [using the method described by Association of Official Agricultural Chemists (AOAC, 1984)], titratable acidity (Gutiérrez-Miceli *et al*., 2007), fruit juice acidity (using pH meter), soluble solid content (using refractometer), and dry matter percent were assayed after harvest.

Statistical analyses

The experiment was arranged in a completely randomized design. Four replicates per treatment were used, each with four plants. Data were analyzed using JMP statistical software, version 5.1 (SAS Institute Inc., Cary, NC, USA). If the interaction was significant, it was used to explain results; if it was not significant, means were separated with Least Significant Differences (LSD) test at $P \leq 0.05$.

3. Results and Discussions

The ANOVA revealed significant main and interaction effects of vermicompost and AMF for most measured characteristics (Table 2).

Determination of plant association with AMF

For the first time in literature, our results report the association of pepino with two AMF. The results indicate that *G. etunicatum* and *G. versiforme* can colonize pepino

Table 1 - Physical and chemical properties of soil and vermicompost used for the experiment

	Sand (%)	Silt (%)	Clay (%)	EC (dS/m)	pH	N (%)	P	K
Soil	34	46	20	1.63	7.82	0.031	5.4 mg/kg	135 mg/kg
Vermicompost	-	-	-	5	8.25	1.45	1.75%	1.2%

roots up to 30 and 50 percent, respectively. It has been stated that the difference between root colonization percentages of *Glomus* strains might be due to the fact that AMF have a wide host range, yet certain combinations of hosts and fungi are more efficient than others for either the fungus or the host (Douds and Millner, 1999; Gutierrez-Miceli *et al*., 2008). van der Heijden *et al*. (1998) showed that plant species differed in their dependency on AMF. Some results suggest that AMF has some degree of host-specificity (Eom *et al*., 2000).

Root colonization

The main and interaction effects of vermicompost and AMF on root colonization percent were significant (Table 2). Root colonization percentages in vermicompost amended soils were about 25% greater than the V_0 treatment regardless of AMF (Table 3). The greater percentage of mycorrhization in vermicompost amended soils has been attributed to the humic substances found in vermicompost, resulting in an increased metabolism of soil microorganisms, and the nutrient uptake (Atiyeh *et al*., 2002).

There was no significant difference between colonized root length of *G. versiforme* and *G. etunicatum* in vermicompost amended soils, but compared to NIC plants, a greater root colonization was observed (2.46 and 2.80 times, respectively) (Table 4). Mycorrhizal plants colonized well with introduced AMF species. A 15% mean root colonization in NIC plants shows that the soil used contained native AMF populations.

More than 100% greater root colonization was observed in the presence of vermicompost when compared with V_0 treatment (Table 5). This could be due to greater organic matter available for growth and development of AMF hyphae; it has been reported that AMF mycelia can mineralize and enhance utilization of organic materials (Feng *et al*., 2003).

Days to first flower formation

The main and interaction effects of vermicompost and AMF on the number of days to first flower formation were significant (Table 2). The number of days from planting to first flower formation was much lower in pots amended with vermicompost than in V_0 treatments (Table 3). Previous studies showing earlier flowering due to vermicompost application on German chamomile, begonia, and coleus (Tomati *et al*., 1983; Tomati *et al*., 1987; Azizi *et al*., 2008) are in agreement with our results. The reason for this has been attributed to the development of efficient photosynthetic structure, higher dry matter production, early initiation, and greater development of the reproductive system (Krishna *et al*., 2008).

Inoculation with *G. etunicatum* gave earlier flowering than *G. versiforme*-inoculated plants (Table 4). In agreement with our results, *Chrysanthemum* cuttings inoculated with AMF had a significantly shorter flowering time compared with non-inoculated plants (Sohn *et al*., 2003). It is reported that in AMF-inoculated tomato plants, the time between emergence and completion of fruit set (the duration of purely vegetative growth) decreased, while the duration of the reproduction period increased (Bryla and Koide, 1998). This is consistent with the idea that plant resource status serves as a partial control of the switch

Table 2 - Analyses of variance for vermicompost application and arbuscular mycorrhizal fungi inoculation on some pepino plant characteristics

Source of variation	df	Mean squares										
		Root colonization	Days to first flower	Flower number in truss	Days from flowering to fruit set	Fruit Number	Fruit fresh weight	Fruit dry matter percent	Fruit juice pH	Fruit titratable acidity	Total soluble solids	Fruit vitamin C content
Vermicompost	1	67**	1056.25**	28.44**	42.25**	27.39**	2321.47**	14.06**	0.06 ns	0.15**	0.13 ns	264.23**
AMF	2	26**	44.62**	0.16 ns	45.42**	4.64 ns	513.87*	90.97**	0.08**	0.21**	2.98 ns	165.20**
Vermicompost × AMF	2	12*	32.92 **	0.75 ns	28.15**	4.99 ns	18.16 ns	28.72**	0.11**	0.12**	5.92*	147.53**
Error	30	0.004	5.08	0.87	1.58	3.46	130.21	0.58	0.02	0.01	1.00	1.56
Total	35											

ns, *, *= non-significant, significant at 0.05 and 0.01, respectively.

Table 3 - The main effects of vermicompost application on some pepino plant characteristics

Treatment	Root colonization percent	Days to first flower	Flower number in truss	Days from flowering to fruit set	Fruit Number	Fruit weight (g)	Fruit dry matter percent	Fruit titratable acidity (ml/100 ml)	Fruit vitamin C (mg/100 ml)
Control	18 b	74.11 a	7.36 b	16.14 a	1.81 b	29.69 b	11.10 a	0.78 a	19.83 a
Vermicompost added	45 a	63.28 b	9.14 a	13.97 b	3.56 a	45.75 a	9.85 b	0.65 b	14.41 b
LSD value at p≤0.05	0.04	1.53	0.63	0.70	1.07	7.77	0.52	0.07	0.85

Values in each column with the same letter are not significantly different using LSD test at p≤0.05.

from vegetative to reproductive growth (Marschner, 1995). In mycorrhizal plants, greater root development leads to more phosphorus in vegetative and reproductive tissues, which eventually leads to early flowering (Bryla and Koide, 1998). This might explain the early flowering in this experiment due to inoculation with *G. etunicatum*.

Plants inoculated with *G. versiforme* and *G. etunicatum* in vermicompost amended pots had 15 and 10 days earlier flowering, respectively, compared to non vermicompost amended pots (Table 5). The organic material provided by vermicompost can improve growth and development of AMF inoculum (Bending *et al*., 2004), which enhances nutrient uptake by the plant and hastens plant growth and development (Mahmood and Rizvi, 2010).

Flower number in truss

A 24% increase in the number of flowers per truss was observed in vermicompost amended pots compared with V_0 treatments, however the effects of AMF and co-application of AMF and vermicompost were not significant (Tables 2, 3). Previously, a 40% increase in flower number in strawberry was related to the increase in plant biological activity due to a vermicompost application rate of over 10 t/ha (Arancon *et al*., 2004 b). A 20% (v/v) vermicompost application in the present study led to a very significant increase in flower number in trusses (Table 2). Some possible factors that improve flowering after vermicompost application have been attributed to the improvement in physical structure of growth medium, increased biological enzymatic activities, increased populations of beneficial microorganisms, or the presence of biologically active plant growth-influencing substances (plant growth regulators) in the vermicompost (Arancon *et al*., 2008). Our results on increased flower number due to vermicompost are in agreement with previous reports on eggplant and tomato (Gajalakshmi and Abbasi, 2002).

Days to first fruit set

The main and interaction effects of vermicompost and AMF on the number of days from flowering to first fruit set were significant (Table 2). Vermicompost amended pots showed fruit set occurring an average of 2.17 days earlier compared with V_0 treatments (Table 3). Non-inoculated control plants and plants inoculated with *G. versiforme* showed fruit set to be three days earlier than in plants inoculated with *G. etunicatum* (Table 4).

The two AMF species showed different interactions with regard to the presence of vermicompost in the medium. Those inoculated with *G. etunicatum* in the presence of vermicompost set fruit 5.58 days earlier compared to non vermicompost amended soil (Table 5). It seems that the potential efficiency of *G. etunicatum* for earlier pepino fruit set is greater in the presence of vermicompost than with *G. versiforme*. NIC plants and plants inoculated with *G. versiforme* with vermicompost added and non vermicompost added media showed no differences.

Fruit number

Our results show that vermicompost application was the primary contributing factor in increasing pepino fruit

Table 4 - The main effects of arbuscular mycorrhizal fungi inoculation on some pepino plant characteristics

Treatment	Root colonization percent	Days to first flower	Days from flowering to fruit set	Fruit weight (g)	Fruit dry matter percent	Fruit juice pH	Fruit titratable acidity (ml/100 ml)	Fruit Vitamin C (mg/100 ml)
NIC	15 b	68.08 ab	13.75 b	32.64 b	8.79 b	5.02 b	0.67 b	16.54 b
Glomus versiforme	37 a	70.85 a	14.12 b	35.41 b	13.65 a	4.99 b	0.87 a	21.08 a
Glomus etunicatum	42 a	67.15 b	17.29 a	45.10 a	8.98 b	5.15 a	0.62 b	13.73 c
LSD value at p≤0.05	5.01	1.88	0.86	9.51	0.63	0.12	0.08	1.04

NIC= Non inoculated control. Values in each column with the same letter are not significantly different using LSD test at p≤0.05.

Table 5 - The interaction effects of vermicompost application and arbuscular mycorrhizal fungi inoculation on some pepino plant characteristics

		Root colonization percent	Days to first flower	Days from flowering to fruit set	Fruit dry matter percent	Fruit juice pH	Fruit titratable acidity (ml/100 ml)	Total soluble solids (brix)	Vitamin C (mg/100 ml)
No-vermicompost added	Non mycorrhizal control	5 c	72.17 b	13.58 b	8.06 d	5.09 ab	0.68 b	7.13 c	22.39 b
	Glomus versiforme	22 b	78.12 a	14.75 b	15.96 a	4.88 c	1.04 a	9.38 a	24.42 a
	Glomus etunicatum	27 b	72.04 b	20.08 a	9.28 c	5.06 ab	0.61 b	7.68 bc	12.66 e
Vermicompost added	Non mycorrhizal control	25 b	64.00 c	13.92 b	9.53 c	4.95 bc	0.65 b	8.15 bc	10.68 f
	Glomus versiforme	53 a	63.58 c	13.50 b	11.35 b	5.10 ab	0.69 b	7.88 bc	17.74 c
	Glomus etunicatum	58 a	62.25 c	14.50 b	8.68 cd	5.23 a	0.61 b	8.52 ab	14.80 d
LSD value at p≤0.05		7.0	2.66	1.21	0.90	0.17	0.12	1.18	1.47

Values in each column with the same letter are not significantly different using LSD test at p≤0.05

number (Table 2). Fruit number in vermicompost amended soils (an average of 3.56) was about 96% greater than V_0 treatments (1.81 fruits) (Table 3). Previously, increased yields of strawberry (Arancon *et al*., 2004 b) and pepper (Arancon *et al*., 2005) in vermicompost amended soils in field conditions were attributed to increased fruit number due to the availability of plant growth regulators and humic acids, which produced by the greatly increased microbial populations resulting from earthworm activity (Arancon *et al*., 2004 a; b). According to our results, the simultaneous increased flower number and fruit set percentage considerably increased total yield.

Fruit fresh weight

The main effects of vermicompost and AMF on fruit fresh weight showed significant differences, but the interaction of vermicompost and AMF was not significant (Table 2). Comparing fruit weight in vermicompost amended soils with V_0 treatments showed a 54% increase (Table 3). This result is in agreement with previous studies on the application of vermicompost for eggplant (Moraditochaee *et al*., 2011), greenhouse pepper (Arancon *et al*., 2004 a), and tomato (Arancon *et al*., 2003). It has been stated that the great microbial activity and populations in vermicompost are probably responsible for a considerable buildup of microbial populations and activity in soils. These improve the soil structure and have an indirect influence on root environment, nutrient absorption, plant growth (Arancon *et al*., 2005), and yield (Goswami *et al*., 2001).

No significant differences were found between plants inoculated with *G. versiforme* and NIC treatments. Plants inoculated with *G. etunicatum* produced fruits with an average weight of 45.1 g, which was about 32% greater than NIC and *G. versiforme*-treated plants (Table 4). It has been reported that individual tomato fruit weight significantly increased when the plants were colonized with AMF (Bryla and Koide, 1998). Such evidence, which is in agreement with our results, also showed different increased levels of fruit fresh weight for other inoculated Solanaceous plants, i.e. tomato plants inoculated with *G. mosseae* (Abdel Latef and Chaoxing, 2010), chili pepper plants inoculated with *G. intraradices* (Castillo *et al*., 2009), chileancho pepper inoculated with *G. fasciculatum* (Mena-Violante *et al*., 2006), and non-Solanaceous cucumber (Trimble and Knowles, 1995). Increased yields have been attributed to the increased yield components due to the positive effects of mycorrhiza including facilitated water and nutrient uptake through extension of root surfaces and increased photosynthesis (Ortas *et al*., 1996; Raman and Mahadevan, 1996; Tarkalson *et al*., 1998).

Fruit dry matter percent

The main and interaction effects of vermicompost and AMF on fruit dry matter percent showed significant differences (Table 2). The V_0 treatments produced 12% greater fruit dry matter than pots amended with vermicompost, regardless of AMF inoculation (Table 3). Results of fruit fresh weight and fruit dry matter percentage showed that vermicompost improved water uptake and partitioning in fruits, which had greater fresh weight (due to vermicompost) and lower dry matter percentages (Table 3).

Inoculating pepino plants with *G. versiforme* increased the fruit dry matter percent to over 50% compared to those inoculated with *G. etunicatum* and plants not inoculated with AMF (Table 4). The differences could be related to the developmental pattern of AMF species. An increased fruit dry matter percentage in AMF plants has been attributed to improved water and nutrient uptake/translocation, higher photosynthesis (Vamerali *et al*., 2003), and also to the pattern of dry matter distribution in inoculated plants, which pointed to a role of AMF in carbon partitioning (Mena-Violante *et al*., 2006).

Co-application of vermicompost and AMF showed different patterns for fruit dry matter percentage. *G. versiforme* in V_0 treatment produced about 40% greater fruit dry matter than vermicompost amended media, but no differences were observed between vermicompost and non vermicompost amended soils inoculated with *G. etunicatum* (Table 5).

Fruit juice pH

With regard to fruit juice pH, the main effects of AMF and its interaction with vermicompost were highly significant (Table 2). The pH of pepino plants inoculated with *G. etunicatum* was 0.13 higher than in NIC plants. The difference between *G. versiforme* inoculated plants and NIC plants was not significant for fruit juice pH (Table 4). Previously, inoculation of cucumber plants with *G. intraradices* gave no changes in fruit pH (Rouphael *et al*., 2010).

Plants inoculated with *G. etunicatum* exhibited a higher fruit juice pH than *G. versiforme* inoculated plants in V_0 treatment, but the difference was not significant when vermicompost was used (Table 5).

Fruit titratable acidity

The main and interaction effects of vermicompost and inoculation with AMF showed significant differences with NIC plants for fruit juice titratable acidity (Table 2). The plants grown in vermicompost amended soils (0.65 ml/100 ml fruit juice) had a 20% decrease in titratable acidity compared with plants in non-amended soils (0.78 ml/100 ml fruit juice) (Table 3). In other studies, the effect of vermicompost on tomato fruit titratable acidity did not show significant differences (Gutiérrez-Miceli *et al*., 2007).

The effect of inoculation of plants with *G. versiforme* showed a 40% increase in fruit titratable acidity compared with *G. etunicatum* inoculated plants and the control (Table 4). Our result is in agreement with a previous paper that reports a significant increase in fruit titratable acidity of AMF inoculated tomato plants (Regvar *et al*., 2003).

A different reaction to vermicompost application was observed for inoculated plants with two different AMFs (Table 5). Fruits of plants inoculated with *G. versiforme* in the V_0 treatment had a 0.5ml/100ml greater titratable acidity than fruits from *G. etunicatum*-inoculated plants.

It seems that the effect of AMF on pepino fruit juice titratable acidity is AMF-species dependent.

Total soluble solids

The main effects of vermicompost and AMF on total soluble solids were not significant but the interaction was significant (Table 2). Fruits of *G. versiforme*-inoculated plants in the V_0 treatment had 1.5 Brix greater soluble solid content than those grown in vermicompost amended media. The differences between AMF-inoculated and NIC plants in vermicompost amended treatments were not significant (Table 5). Greater fruit total soluble solids in AMF inoculated tomato plants, compared to non-inoculated plants, has been previously reported (Subramanian *et al*., 2006). Different microorganisms have been reported as involved in breaking down (mineralize) and releasing mineral nutrients of organic materials, to then be taken up by plant roots (Linderman and Davis, 2004). It seems that *G. etunicatum* has the ability to improve vermicompost utilization by pepino plant roots, which leads to more efficient photosynthetic activity and therefore greater total soluble solids in fruits.

Fruit vitamin C

The main and interaction effects of vermicompost and inoculation with AMF showed significant differences for fruit vitamin C content (Table 2). Fruits produced in vermicompost amended soils had 37% less vitamin C than fruits in the V_0 treatments (Table 3). This could be related to a 1.5 times greater fresh fruit weight with constant vitamin C content in a unit volume. It seems that the level of vitamin C in pepino fruits is not affected by vermicompost application. Different reports are available on the effect of vermicompost application on fruit ascorbic acid content: some show increased fruit vitamin C in tomato (Sable *et al*., 2007), while others report no significant effect (Roberts *et al*., 2007).

The fruit vitamin C content was 53 and 27% greater in fruits from plants inoculated with *G. versiforme* as compared to fruits from plants inoculated with *G. etunicatum* and non mycorrhizal treatments, respectively (Table 3). Higher quantities of ascorbic acid in AMF-inoculated tomato plants compared to non-inoculated plants has been previously reported (Subramanian *et al*., 2006).

The AMF used in this experiment showed different reactions to vermicompost application. Fruits from the V_0 treatment inoculated with *G. versiforme* and those inoculated with *G. etunicatum* in vermicompost amended soils had greater vitamin C than other treatments (Table 5). It seems that the effect of AMF on pepino fruit vitamin C is species dependent. Those plants treated with *G. etunicatum* had higher vitamin C content when vermicompost was applied, while the same trend was not observed when vermicompost was applied to *G. versiforme*-inoculated plants.

4. Conclusions

Despite the influence of AMF on crop yield as documented in many reports on Solanaceous plants, little is known about the potential of AMF to improve their fruit quality. Crop species and cultivars of plant species can differ dramatically in their ability to respond to different AMF strains. This can complicate predictions of the extent to which AMF colonize roots and the resulting effects on plant growth and development. This is the first report on the effects of AMF inoculation in pepino, on the level of colonization, plant growth, development, and fruit yield and quality. We have clearly demonstrated the positive impact of AMF on pepino fruit quality in terms of vitamin C, total acidity, pH, total soluble solid content, increased titratable acidity and dry matter percentage.

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Establishment of a cell suspension culture of the halophyte *Cakile maritima*

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Keys words: biofuel, *Cakile maritima*, cell suspension, halophytes, oil.

Abstract: *Cakile maritima* is a member of the Brassicaceae family also known as sea rocket. It is an annual succulent halophyte frequent in coastal dune vegetation in Mediterranean regions and Atlantic coasts from North Africa to the north of Europe. This halophyte presents a complex survival strategy at high salinity and its seeds contain up to 40% of an oil which could be suitable for biofuel production and other industrial applications. However, data concerning the cellular mechanisms allowing this plant to resist salinity are still lacking. Cell suspension cultures offer an *in vitro* system convenient for cell biology studies and biotechnological methods are still not developed for this putative crop. The present paper reports initiation of *C. maritima* cell suspension cultures from callus obtained from aerial parts of seedlings. The establishment of a suspension culture which preserves its salt resistance provides an opportunity to gain insights into *C. maritima* biology.

1. Introduction

The world's cultivated lands are increasingly affected by drought and salinity (Barrett-Lennard, 2000). Halophytes growing in highly saline soils could thus serve as a resource for the identification and development of new crop systems for marginal saline soils (Debez *et al*., 2011; Ben Hamed *et al*., 2013). The actual yield of halophytes remains largely unknown since their domestication is still limited, yet the economic potential of some of these plants has been positively assessed by various groups (Aronson, 1989; Ashour and Thalooth, 1993; Abdelly *et al*., 2006). Numerous Brassicaceae species are current and emerging biodiesel crops: in addition to the oil-rich seed, the ability of Brassicaceae species to grow on marginal land with minimal inputs make them particularly attractive and potentially viable for this application. Among halophyte species studied for their potential as oleaginous plants, the Brassicaceae *Cakile edentula* (O'Leary *et al*., 1985), *Crambe abysinnica* (Mandal *et al*., 2002) or *Cakile maritima* (Ghars *et al*., 2005) have been reported to contain high amounts of oil. *Cakile maritima*, the sea rocket, is an annual succulent halophyte frequently found from the Black Sea coasts to the Mediterranean region, and from the Atlantic coasts of North Africa to the north of Europe (Clausing *et al*., 2000). Tunisian accessions of *C. maritima* contain up to 40% seed-oil (Ghars *et al*., 2005). Plant growth, harvest index, silique number and seeds produced per fruit segment is maximal at 100 mM NaCl (Debez *et al*., 2008) but *C. maritima* can survive at up to 800 mM NaCl (Ellouzi *et al*., 2013) and successfully reproduces till 500 mM NaCl salinity (Debez *et al*., 2004). Seed-oil content did not seem to be affected by salinity, although erucic acid level could increase (Debez *et al*., 2006). These facts highlight the need to better understand the basis of adaptation to saline environments, as well as traits associated with oil production itself. In previous works we described some aspects of the response of *C. maritima* to salt stress (Debez *et al*., 2004, 2006, 2008; Ellouzi *et al*., 2011) and our data point out that *C. maritima* adopts a complex survival strategy at high salinity, however numerous data concerning the cellular mechanisms allowing this plant to resist salinity are still lacking.

Suspension culture cells offer an *in vitro* system that is widely used in plant biology as a convenient tool to investigate a wide range of phenomena. It consists in a model system as suspension culture provide a ready source of a homogenous cell type and avoids the complications of multicellular tissue types *in planta* (Moscatiello *et al*., 2013). Suspension culture cells were recently used in various studies and models concerning plant responses to salinity, such as proteomic studies (Chen *et al*., 2012; Liu *et al*., 2013), metabolomic studies (Liu *et al*., 2013) or

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transcriptomic studies (Matsuura *et al*., 2010; Bae *et al*., 2012). It is also a convenient means to study transport system regulations and oxidative responses to various biotic and abiotic constraints (Kadono *et al*., 2010; Baz *et al*., 2012; Yukihiro *et al*., 2012; Tran *et al*., 2013), comprising salinity (Cessna *et al*., 2007; Wang *et al*., 2010; Pons *et al*., 2011; Queirós *et al*., 2011).

Here, we report the development of a cell suspension culture of *C. maritime*; growth performance was evaluated on control and NaCl cultured cells to validate the biological system. These suspension culture cells could be a valuable tool to gain further insights into halophyte studies and their potential applications.

2. Materials and Methods

Establishment of callus of Cakile maritima

In this study we used *Cakile maritima* seeds harvested in the Raoued region in the north of Tunisia. Callus cultures were initiated from the aerial part of 14-day-old, light-grown seedlings. Seeds were submerged in 70% ethanol for 1 min, then rinsed with sterile distilled water, submerged in chlorine bleach for 10 min and then rinsed three times (5 min each) with sterile distilled water. The seeds were placed in petri-dishes containing Murashige and Skoog medium including vitamins (MS) (Murashige and Skoog, 1962), supplemented with 30 $g.L^{-1}$ sucrose, 8 $g.L^{-1}$ agar. The pH was adjusted to 5.8 with KOH.

Stem segments were finely cut and then placed on a solid callus-inducing medium (CIM) containing 6.2 $g.L^{-1}$ Gamborg B5 (Gamborg *et al*., 1968) supplemented with 20 $g.L^{-1}$ glucose, 8 $g.L^{-1}$ agar and with growth regulators 9.06 µM of 2.4 D and 0.46 µM of kinetin. The pH was adjusted to 5.7 with KOH. After two to three weeks, callus appeared on the sides of the segments. When the size of callus became larger than 1 cm, they were divided and transferred to a new medium.

Establishment cell suspension cultures of Cakile maritima

Approximately 5 g of callus were transferred to 125 mL flasks containing fresh Gamborg B5 medium supplemented with 30 $g.L^{-1}$ glucose, 0.2 µM 2,4-D and 0.45 µM kinetin. The pH was adjusted to 5.7 with KOH. Flasks were incubated on a rotary shaker at 120 rpm and maintained at 22°C in the dark. Feeding of the cultures with fresh medium was done at 10-14 day intervals during which time the suspensions were allowed to settle under agitation (rotary shaker at 120 rpm). This procedure was repeated for about eight weeks. Then, the suspension was subcultured every seven days by transferring 20 mL of the culture into 50 mL of fresh medium in 250 mL Erlenmeyer flasks.

Arabidopsis thaliana *cell suspension culture conditions*

Arabidopsis thaliana L. cell suspensions were prepared from calluses of the cell line T87 generated from the ecotype Columbia plant as previously described (Tran *et al*., 2013). The suspension cells were obtained after about two months and five to six subcultures in 1 L round-bottom flasks containing 350 ml liquid Gamborg B5 culture medium (pH 5.8). Cell suspensions were sub-cultured weekly using a 1:10 dilution.

Growth evaluation

Growth of the culture was evaluated by measuring the fresh weight of cells and the density of cells using a Nageotte cell.

Cell viability

Cell viability was assayed using the vital dye neutral red. Cells (100 µL) were incubated for 5 min in 400 µL phosphate buffer pH 7 with neutral red to a final concentration of 0.001% (w/v). Cells that did not accumulate neutral red were considered dead. At least 500 cells were counted for each replicate and the procedure was repeated at least three times for each treatment.

External pH measurements

Measurements of extracellular pH were performed with pH-sensitive electrodes every 24 h for six days from 5 mL of cultured medium cells. The procedure was repeated on three independent subcultures.

Cell size

Cell images were recorded with a camera (Kappa CF11DSP) on a light microscope (Labophot-2 Nikon) and sizes were measured using image analysis software KappaImageBase-2.2SP2-Metreo (Kappa Optoelectronics GmbH, Gleichen, Germany).

Protoplast Isolation

Protoplasts were isolated from suspension cultures six days after subculture. Fifteen mL of suspension cells were used. After cell sedimentation, the supernatant was removed and replaced by 5 mL of Gamborg B5 fresh medium containing 0.1 g cellulysin, 0.05 g macerase and 0.3 M sorbitol. The digestion was carried out under shaking at 120 rpm at 22°C for 30 min. After incubation, protoplasts were collected by centrifugation at 300 rpm for 3 min and re-suspended in 5 mL of Gamborg B5 fresh medium supplemented with 0.6 M sorbitol or 0.3 M sorbitol.

3. Results and Discussion

The different steps in the development of *Cakile maritima* callus from stem segments are reported in figure 1. Callus appeared on the sides of the discs after two to three weeks. Although produced in light, callus became non-chlorophyllian and pale yellow in color (Fig. 1C, D). Thus *Cakile maritima* loses its power of chlorophyll synthesis during its passage from plant stage to callus stage. To generate cell suspension, approximately 5 g of calluses were transferred in Gamborg medium. These calluses progressively disintegrated to the smallest cell aggregates in the liquid medium.

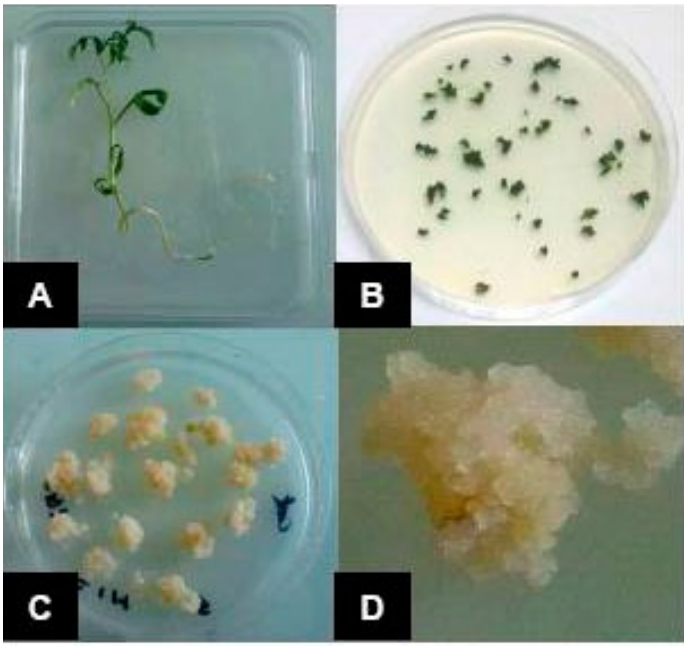

Fig. 1 - Establishment *of Cakile maritima* calluses. (A) Three-week-old *C. maritima* seedling grown from sterilized seed. (B) Slices of leaf and rod were finely cut for callus generation. (C) One-month-old calluses derived from rod and leaf slices. (D) Magnification of a callus.

Large quantities of cells could be obtained after two months feeding (Fig. 2A, B). At this stage suspension cells were subcultured every week in fresh Gamborg medium and after an additional 1.5 months the culture became more homogeneous as the cell aggregates became smaller and the cell size increased (Fig. 2C, D). The level of dead cells in the culture also progressively decreased reaching about 10% after four months of culture, in the same range as observed for tobacco or *A. thaliana* suspension cells (Baz *et al*., 2012; Yukihiro *et al*., 2012; Tran *et al*., 2013).

Using four-month-old *C. maritima* culture, growth curves were established by measuring the fresh weight of cells (Fig. 3A) and the cell density (Fig. 3B). Both curves showed a typical sigmoidal shape with a latency period of 24 h, then an exponential phase lasting about four days prior to a plateau phase (Fig. 3A, B) supposed to be due to nutrient depletion. We monitored the medium pH during the culture procedure (Fig. 3C). After a slight acidification at the beginning of the exponential phase, the pH became more alkaline, reaching 6.5 at the end of the culture (Fig. 3C). This alkalization could be involved in the decrease in biomass production as Ling *et al*. (2008) pointed out that pH of 6.7 lowered the growth of suspension culture of *Ficus deltoidea*. However, the pH variations recorded during the culture should remain suitable for nitrate uptake as reported for *Ipomoea* suspension cells (Martin and Rose, 1976).

Histological analysis on six-day-old suspension cells revealed distinct morphological features of the cultured cells (Fig. 4). Although the cell aggregate size diminished during culture establishment, a few large aggregates (>20 cells) remained. However the largest part of the culture consisted of compact small groups of less than 20 cells, 60% corresponding to groups of three to 10 cells, single cells representing about 10% of the population (Fig. 4A). Most of the cells were rounded in shape (Fig. 4B, left and center); less than 2% were elongated (Fig. 4B, right). Interestingly, as observed during the establishment of the culture (Fig. 2B, D), the size of the cells seemed to be dependent on the size of the groups, the isolated cells being the largest (Fig. 4C).

It is known that protoplasts can be used for transient expression, trafficking assay or ion homeostasis analysis, notably in studies on plant resistance to salinity (Laohavisit *et al*., 2012; Haro *et al*., 2013; Morgan *et al*., 2013; Mottaleb *et al*., 2013; Son *et al*., 2013). Thus, we evaluated the usefulness of the cell cultures for the isolation of protoplasts. Protoplasts were isolated from six-day-old suspension cultures. Based on the different cell sizes measured (Fig. 4B, C) we observed protoplasts of different sizes. Most of these protoplasts maintained in 0.6 M sorbitol ap-

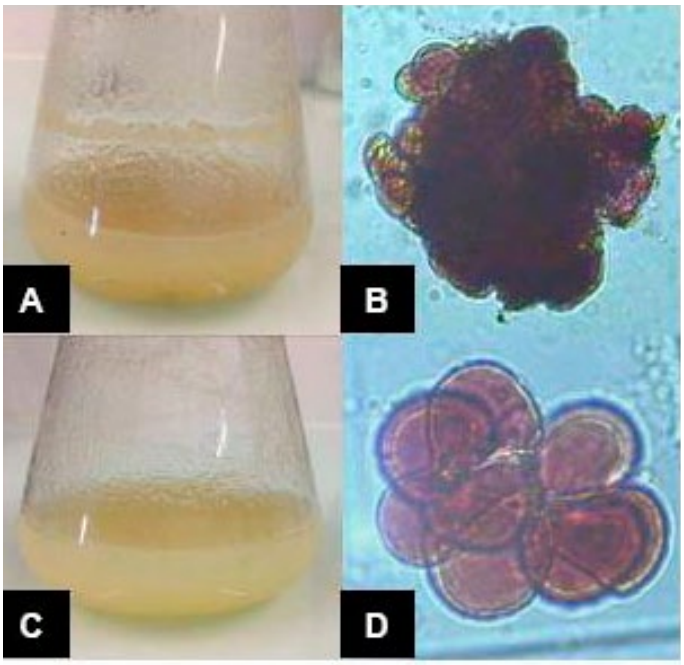

Fig. 2 - Establishment *of Cakile maritima* cell suspensions. (A) Dense suspension cells grown in flask after two months feeding and (B) corresponding cell aggregates magnified 385X. (C) Dense suspension cells grown in flask after 1.5 months of subculture after feeding and (D) corresponding cell aggregates magnified 385X.

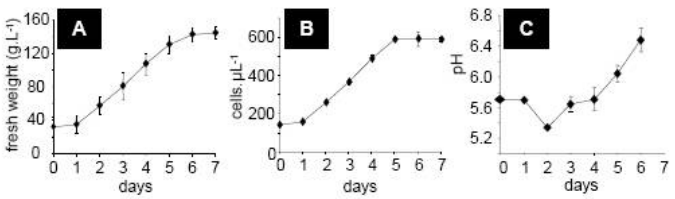

Fig. 3 - Growth pattern of *Cakile maritima* cell suspension determined by fresh weight (A) or cell density measurements (B). Evolution of the medium pH during cell suspension growth (C). Bars indicate mean ± SD of at least three experiments.

peared plasmolyzed and numerous protoplasts seemed to be shrunken (Fig. 5A). We then used 0.3 M sorbitol and obtained rounded protoplasts with large vacuoled evidenced by neutral red staining (Fig. 5B).

Finally, *Cakile maritima* being a halophyte, we checked the salinity resistance of the suspension cells. Suspension cells were subcultured in Gamborg medium complemented with 100, 400 or 800 mM NaCl. Sigmoidal growth curves were obtained and no significant differences were observed between the control and the suspension cells growing in presence of 100 mM NaCl when 400 mM and 800 mM strongly decreased the growth of the suspension cells (Fig. 6A). These data are in accordance with what was described for seedlings, *C. maritima* even requiring the presence of a moderate salt concentration (50-100 mM NaCl) to maintain a significant growth activity and plant development (Debez *et al*., 2004, 2008). Although strongly reduced at 400 mM or 800 mM NaCl, the growth of the suspension cells was also in accordance with previous data indicating that *C. maritima* can survive up to 800 mM

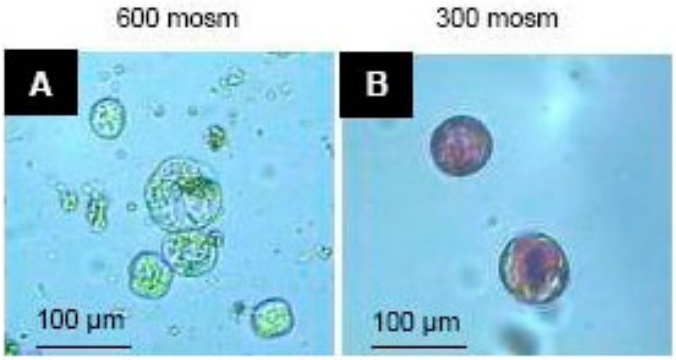

Fig. 5 - Protoplasts derived from six-day-old *Cakile maritima* cell suspension maintained in 0.6 M sorbitol (A) or 0.3 M sorbitol (B).

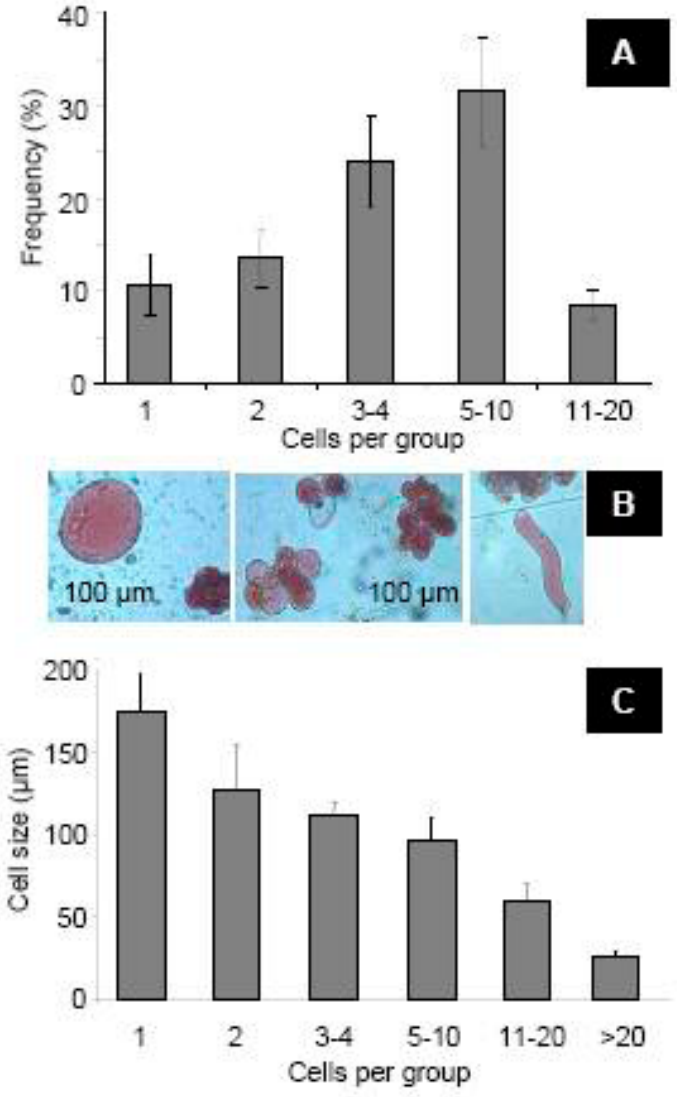

Fig. 4 - Morphology of six-day-old *Cakile maritima* suspension cells. (A) Frequency of cell groups in the culture. (B) Different morphologies of cells. (C) Sizes of the cells according to the size of the cell groups. At least 250 cells were analyzed; bars indicate mean ± SD.

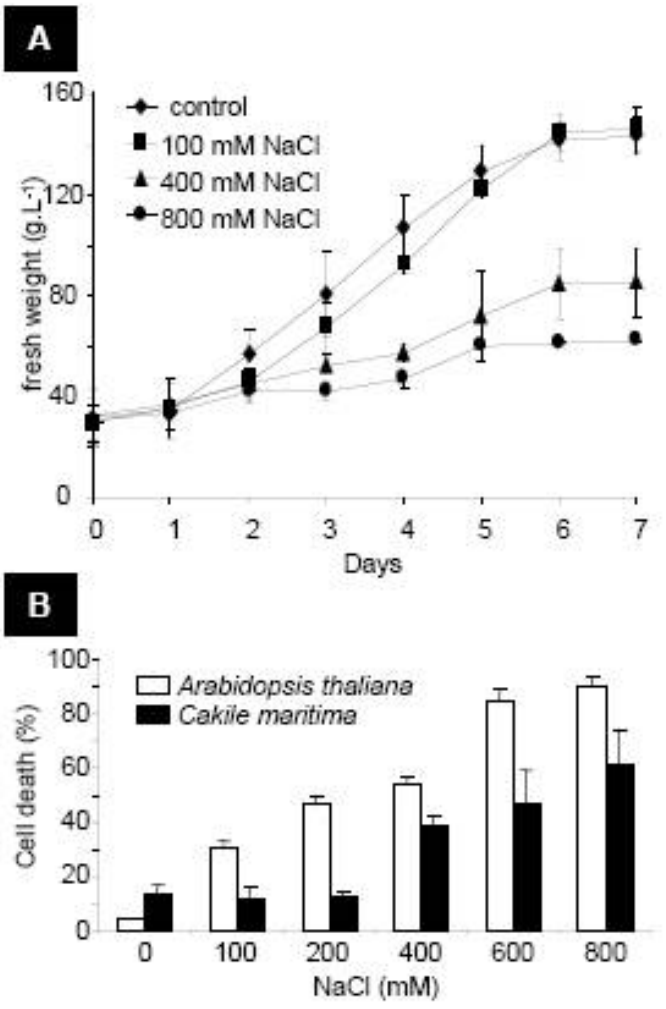

Fig. 6 - Evaluation of *Cakile maritima* cell suspension resistance to salinity. (A) Growth pattern of cell suspensions grown in presence of 100, 400 or 800 mM NaCl determined by fresh weight measurement (B). Comparison of cell death extents of *C. maritima* and *Arabidopsis thaliana* cell suspensions treated 6 h with NaCl concentrations ranging from 100 to 800 mM. Bars indicate mean ± SD of three experiments.

NaCl (Ellouzi *et al*., 2013) and successfully reproduces till 500 mM NaCl salinity even if the biomass was reduced (Debez *et al*., 2004). We further compared the extent of cell death induced 6 h after the addition of various NaCl concentrations on *C. maritima* and *A. thaliana* suspension cells. Cell death began to increase from 100 mM NaCl to almost 100% at 600 mM for *A. thaliana* (Fig. 6B). For *C. maritima*, the increase in cell death was significant only with 400 mM NaCl and reached only 60% at 800 mM, remaining largely inferior when compared to *A. thaliana* (Fig. 6B). It is worth noting that 40% of surviving cells probably go on dividing, which explains the growth of the culture, although reduced, at 800 mM NaCl (Fig. 6A). As a whole, these data demonstrate that *C. maritima* suspension cells preserve their ability to resist salinity.

4. Conclusions

The present study reports the development of *C. maritima* cell suspension cultures which maintained their salt resistance, offering greater understanding about adaptation to saline environments, as well as traits associated with biofuel production.

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