

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

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

1. Introduction

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

(Davies *et al.*, 1993). Plant adaptations to arid climate conditions, morphological and physiological changes, and concentration of novel metabolites along with structural variations, increase their efficiencies in stress conditions (Wu *et al.*, 2006).

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

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biomass while they reduced their colonization percentage. These increases were attributed to the improvement of fungi water absorptions and increases in the length and volume of plant fungal roots (Faber *et al.*, 1991; Bryla and Duniway, 1997; Wu *et al.*, 2011). In these plants, root colonization increases with a decrease in drought stress (Augé, 2001). Under drought stress conditions, *G. versiforme* fungi increased fresh and dry weight of plant roots and shoots and increased the root colonization percentage (Wu and Xia, 2006). *G. intraradices* fungus, under drought stress conditions, increased root growth and respiration rate of Rough lemon (Leyv and Syvestern, 2006). Many studies showed that *G. etunicatum* fungi could affect plant-water relations of host plants including citrus, under both drought stress and enough-water-supply conditions (Wu *et al.*, 2006). Therefore, they cause higher water use efficiency and this water use efficiency in mycorrhizal plants becomes even more tangible in drought stress conditions (Davies *et al.*, 1993). *Glomus etunicatum* fungus increased phosphorus, potassium, zinc, and copper in pistachio trees planted under sufficient water supply conditions and also increased nitrogen and calcium in pistachio trees planted under drought stress conditions. However, this fungus did not change the magnesium concentration (Abbaspour *et al.*, 2011). It is reported that *Pseudomonas* bacteria enhances growth and yield of some plants (Rodriguez and Fraga, 1999). Construction of active metabolites such as vitamins, amino acids, and Indole acetic bacteria may have a direct effect on the growth and metabolite contents of *Piriformospora indica* and mycorrhizal fungi. As a helpful microorganism, it seems that bacteria supports fungal performances (Vivas *et al.*, 2003). Plant inoculations with different types of *Pseudomonas* bacteria in drought stress situations increased plant proline contents, thus the plants' water levels were maintained and their protein contents and membranes remained safe from drought stress damage (Yoshida *et al.*, 1997). Inoculation with *Pseudomonas* species, led to moderation of drought stress effects, improvement of plant growth and increase of proline, soluble sugars and amino acids production, explaining their effectiveness in absorbing water and nutrients from the soil (Wu *et al.*, 2008). These types of bacteria also help the plant maintain its RWC and LWL (leaf water loss) levels under drought stress conditions. Studies have shown that mycorrhizal plants absorb more CO₂ in the presence of light. Hence, their photosynthesis rates are

also higher. The increase of CO₂ absorption in mycorrhizal plants is related to a decrease of liquid-phase resistance of mesophyll cells to CO₂ transmission (Wu and Zou, 2009). Miller (2000) reported that in mycorrhizal plants, due to the increase of photosynthesis materials and rate, water use efficiency increased per water use unit. Mycorrhiza can increase plant weight, leaf area, and plant pigments, and these increases may be attributed to the improvement of fungi water and phosphorus absorptions (Bethlenfalvai *et al.*, 1988; Davies *et al.*, 1993). *Glomus etunicatum* and *Pseudomonas* bacteria have positive effects on plant growth and employing them, instead of fertilizers, is considered a positive approach to reduce fertilizer use (Davies *et al.*, 1993). Despite the lack of comprehensive scientific investigations on the horticultural characteristics of Mexican lime (*Citrus aurantifolia* Swingle cv. Mexican Lime) as a rootstock, its seed availability for propagation and some its characteristics, such as good crop load and vigorous habit of grafted cultivars as scion, have made it a favorite in Fars province, Iran.

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

2. Materials and Methods

Preparation and inoculation of plant materials

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

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

The Kormanik and McGraw method (Kormanik and McGraw, 1982) was used to measure colonization percentage. In this method, 2 g of roots previously stored in FAA (formaldehyde - acetic acid - ethanol) were washed with water three or four times and were placed in Falcon tubes containing 10% KOH solution for 24 h at room temperature. The color of the solution was almost yellow or light yellow. The solution was then poured out and the roots were again washed with water three or four times. The samples were placed in 2% hydrochloric acid for at least 15 min for staining. The acid was poured out

and a colored solution was poured over the acidic roots. Acid fuchsin stain was used in this study; the ratio of the fuchsin acid colored solution was 14 ml lactic acid, 1 ml glycerin and, 1 ml water. The roots and the solution were kept at room temperature for 24 h. The coloring solution was then removed. Besides, due to elimination of extra colors, the coloring solution was poured on the roots. After 6-12 h, fungal organs such as arbuscules, hyphae, and vesicles were observed under a light microscope and colonization was calculated as a percentage. After application of water stress treatments, leaf chlorophyll content was measured with a SPAD-502 chlorophyll-meter using three fully-expanded leaves to find an average for chlorophyll content. Leaf temperature factors, net photosynthesis and transpiration rates were measured by portable photosynthesis meter (LCi, ADC, England). Relative water content was determined by using ten 7 mm-diameter leaf discs. Leaf discs for each treatment were weighed (FW). They were hydrated until saturation (constant weight) for 48 h at 5°C in darkness (TW). The leaf discs were then dried in an oven at 105°C for 24 h (DW). Relative water content was calculated according to the following expression (Filella et al., 1998):

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

Statistical analysis

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

3. Results and Discussion

Leaf relative water content (RWC)

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

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

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

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

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

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

Chlorophyll content

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

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

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

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

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

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

Leaf temperature

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

Rate of net photosynthesis

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

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

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

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

gation periods, respectively. The results also indicated that the presence of *G. etunicatum* fungi and *Pseudomonas fluorescense* bacteria increased the plants' rate of net photosynthesis. Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescense* bacteria identified that the maximum rate was observed in simultaneous inoculation of both fungi and bacteria and with a two-day irrigation period (12.3 micromole/m²/s) (Table 4).

Table 4 - Effects of *G. etunicatum* fungus and *Pseudomonas fluorescense* bacteria inoculations on Mexican lime photosynthesis rate with different irrigation periods (micromole/m²/s)

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

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

Rate of transpiration

Analysis of the effects of interaction between inoculation of *G. etunicatum* fungi and *Pseudomonas fluorescense* bacteria on Mexican lime transpiration rate in plants grown with different irrigation periods identified that the highest rate was observed in simultaneous inoculation of both fungi and bacteria and a two-day irrigation period (10.25 micromole/m²/s). Likewise, the minimum transpiration rate was observed in the treatment without fungi and bacteria inoculations and a six-day irrigation period. The overall results showed that the leaf tran-

spiration rate increased with the increase of irrigation period (Table 5).

Table 5 - Effects of *G. etunicatum* fungus and *Pseudomonas fluorescense* bacteria inoculations on Mexican lime transpiration rate with different irrigation periods (micromole/m²/s)

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

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

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

Root colonization percentage

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

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

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

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

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

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

4. Conclusions

The results of the current study and other research projects in this field have shown the practi-

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

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