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Effect of arbuscular mycorrhizal fungi and *Pseudomonas fluorescence* on chlorophyll fluorescence and photosynthetic pigments of pistachio seedlings (*Pistacia vera* cv. Qazvini) under four water regimes

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ABSTRACT

To study the interaction effect of arbuscular mycorrhizal fungi (AMF) Glomus mosseae and Pseudomonas fluorescens P_{52} strain bacteria, on chlorophyll fluorescence and photosynthetic pigments of pistachio seedling (Pistacia vera cv. Qazvini) under water stress, a greenhouse experiment was conducted using four levels of water stress (%100 field capacity (FC) as control and 75, 50 and 25% FC) and four levels of biofertilizer (plant without mycorrhizae and bacteria as control, mycorrhizae alone, bacteria alone and mycorrhizae and bacteria combination) in a completely randomized design as factorial with four replications. The minimum amount of maximal quantum yield of photosystem II (PSII) photochemistry (F_v/F_m) was observed in water stress from 25% FC. The use of biofertilizer in water stress from 25% FC increased F_v/F_m. The minimum amount of F_v/F_m in water stress from 50% and 25% FC was observed in last time of measurement. The minimum amount of F_v/F_m in all levels of biofertilizer was related to last time of measurement. Water stress reduced chlorophyll (Chl) and carotenoids (Car) pigments content and the minimum amount of pigments in the combined treatment was observed in water stress from 25% FC. The results of this study showed that the pistachio plant is tolerance to water stress, because reduce in the efficiency of the photosynthetic apparatus occurred in water stress 25% FC and in last time of measurement.

Keywords: Chlorophyll fluorescence, Mycorrhizae, Pigment, Pistachio, Pseudomonas fluorescent bacteria, Water stress.

INTRODUCTION

Pistachio is a subtropical fruit and has become one of the dominant crops in south-east regions of Iran especially in Kerman province [30]. Although pistachio nut tree is one of the crops which have drought tolerance [31], it does not mean that pistachio trees require less water for optimal performance. One way in recent years to deal with water stress which has been used in many plants is using root symbiotic fungi (mycorrhizae) [13]. There are reports that show mycorrhizal fungi symbiosis with the roots of pistachios is made [5, 27]. However pistachio is reputed for its drought tolerance, limited information is available on its mechanisms of drought tolerance associated with photosynthesis especially in combination with arbuscular mycorrhizal fungi (AMF) symbiosis [4]. In recent years, Chl fluorescence analysis has become ubiquitous in plant ecophysiology studies [35]. Chl fluorescence parameters are commonly used. These permit evaluation of parameters that correlate with net CO_2 assimilation (A), such as the quantum yield of PSII, which in turn gives estimates of relative electron transport rate (ETR), provided the light absorbed by the leaf is known [8]. Ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) is used as maximal quantum yield of PSII photochemistry (F_v/F_m). The F_v/F_m in plants grown in suitable conditions is around 0.8 and decrease to 0 under stress conditions. Therefore, in order to demonstrate the tolerance of plants to water stress, F_v/F_m is a very good parameter [29]. Photosynthetic pigments are mainly important to plants for harvesting light and production of reducing powers (ATP, NADPH) [1]. Water stress makes changes in the ratio of Chl *a* and *b*

and Car [2, 7]. Car is a large class of isoprenoid molecules which are synthesized by all photosynthetic and many non-photosynthetic organisms [1]. However, Car has additional roles and partially helps the plants to withstand adversaries of drought [1]. Often useful free living bacteria of rhizosphere are called plant growth-promoting rhizobacteria (PGPR) such as Pseudomonas fluorescent [11]. These bacteria can have a beneficial effect on plant growth with direct (with production of auxin, ACC deaminase enzyme, phosphate solvers, siderophore, salicylic acid, chitinase) and indirect (with remove or regulate harmful effects of pathogenic microorganisms) ways [11]. As regards of the application of PGPR on pistachio alone or in combination with AMF, no report is available. Therefore the present experiment was carried to establish water stress tolerance of Qazvini pistachio seedlings aspect of chlorophyll fluorescence and photosynthetic pigments using the mycorrhizal fungi and *Pseudomonas fluorescens.*

MATERIALS AND METHODS

Experimental site: A greenhouse experiment was conducted between the years of 2010 to 2011 at the Agricollege of Vali-e-Asr university of Rafsanjan (30°23′06″N, 55°55′30″E), at 1,523 m a.s.l.

Preparation of AMF inocula: The AMF that was used in this study was originally recovered from pistachio (*Pistacia vera* L.) orchards grown in different sites of Rafsanjan, Kerman, Iran. These native isolates [*Glomus mosseae* (Nicolson and Gredemann)] were maintained and propagated in sorghum (*Sorghum spp.*) pot cultures using autoclaved soil (as it is described below) in a greenhouse (Tmax: 35 ± 3°C; Tmin: 27 ± 2°C; RH: 55 ± 3%) and adequate amount of sterilized water was supplied every day during the period from August to December 2010. At the end of the period, the shoots of the sorghum plants were removed and substrate was allowed to dry for a week. The roots were finely chopped and the dried root/soil mixture was thoroughly mixed to obtain a homogenous inoculum. Samples for mycorrhizal assessment were prepared according to method of Phillips and Hayman [23] and mycorrhizal colonization (abundance of hyphae, vesicles, and arbuscules) was estimated according to Giovannetti and Mosse [10] at 100 × magnification using 40 root segments of each sample.

Preparation of bacterial suspension: Pistachio seedlings were inoculated with the bacterial suspension of *Pseudomonas fluorescens* strain P_{52} that have been isolated from the rhizosphere of pistachio seedlings. To preparation of bacterial suspension, the bacterium was plated on King B medium. Then, a single colony was infusioned into 20 ml of the TSB1/2 medium with a concentration of 1.2 mg l^{-1} and was shaked at 150 rpm for 24 h. 50 ml of the bacterial suspension was dumped in 10 erlenmeyer flask containing 50 ml of the TSB1/2 medium and were shaked at 150 rpm for 48 h. The obtained suspension was used to inoculate treatments containing bacteria.

Soil preparation and seed sowing: A sandy loam soil was sterilized by autoclaving (121°C, 1 h) on 3 consecutive days in order to eliminate the indigenous endophytes. For each pot a rate of 2.4 g of powdered rock phosphate as a source of insoluble phosphorus in the soil was uniformly mixed. The major characteristics of the soil were as follows: sand 74%, silt 10%, clay 16%, pH 7.9, N 0.119%, P 5 mg kg^{-1} soil, K 139.4 mg kg^{-1} soil, Fe 2.87 µg g^{-1}, Zn 1.18 µg g^{-1}, Mn 2.7 µg g^{-1}, Cu 0.62 µg g^{-1}, and cation exchange capacity 1.7 ms. Seeds of pistachio (*Pistacia vera* cv. Qazvini) were incubated at 30°C on sterile moist cloth for one week. Six germinated seeds were sown into plastic pots containing 4.6 kg of autoclaved soil. The number of seedlings per pot was reduced to 3 within 21 days of germination.

Mycorrhizae and bacteria treatment: The inocula consisted of soil, sorghum chopped root fragments, spores and hyphae colonized with fungi. 100 g of fresh mass (FM) of inoculum having on average of 75% of infected roots was placed on the soil surface and after placing the 6 germinated seeds on it, seeds were covered with sterilized sand. Control plants received the same amount of an autoclaved mixture of inocula. In the bacteria treatment, 2 ml of the suspension of bacteria was poured on the germinated seeds with using a sampler. Moreover the germinated seeds were placed for 30 minutes in bacterial suspension until bacteria penetrate well into the seed. Both treatments of mycorrhizae and bacteria were used in the combination treatment. Before starting the water stress treatments, the seedlings were watered every day up to FC level with distilled water during 90 days. Before starting the water stress treatments to ensure of colonization, the pistachio seedlings roots were randomly sampled. 80% of infection was detected in laboratory.

Irrigation treatments: The four water regimes with 100% FC as control, 75% FC (mild stress), 50% FC (moderate stress), and 25% FC (severe stress). For determination of soil moisture content in FC, pots were saturated and kept for 48 h to let the gravimetric water become drained and then pots were weighed. The difference between pot mass after 48 h with initial pot mass (before saturation) was considered as soil water content in FC. In 100% FC treatment, individual pots were weighed, water added to bring the soil to the FC. For 75, 50, and 25% FC treatments, pots received 75, 50, and 25% of water added to the 100% FC treatment, respectively. Thereafter, for 80 days (from 1 June to 19 August 2011), soil water contents were determined by weighing the pots daily and water was added

following at the time of weighing to maintain the predetermined water content in each pot. The maximum average temperature was 35°C, the minimum average temperature was 27°C and the average relative humidity was 55%.

Chl fluorescence: During 80 days of stress Chl fluorescence parameters were measured in 5 times T_1: 20 days after starting the water stress, T_2: 34 days after starting the water stress, T_3: 50 days after starting the water stress, T_4: 65 days after starting the water stress and T_5: 80 days after starting the water stress. Chl fluorescence parameters were measured with a portable handy *Plant Efficiency Analyzer* (*PEA, Hansatech Instruments Ltd*., UK). Three leaves were selected from each pot and pre-adapted to dark period for 20 min by fixing special tags on each leaf blade before measurements were taken. During dark adaptation, all the reaction centers are fully oxidized and available for photochemistry and any fluorescence yield is quenched. After 20 min of dark adaptation, the sensor cup was fitted on the leaf for measurement (Strasser et al., 2000).

Leaf area: Leaf areas were scanned with using a CI 202 model leaf area meter. Leaf areas were obtained according to Cm^2.

Chl and Car content: Pigments were determined according to Lichtenthaler [16]. At the end of the experiment, 3 fully extended leaves from each pot (one leaf from each plant) were collected and wrapped in aluminium foil to avoid degradation of pigments by light. The extract was prepared from fresh leaves (1 g) by grinding in a cold mortar and pestle together with 10 ml of 80% aqueous acetone. After filtering, absorbance of centrifuged extracts was measured at 663, 645, and 470 nm using a spectrophotometer (*U-2000,Hitachi Instruments*, Tokyo, Japan).

Experimental design: Two-factors factorial experiment (water stress and biofertilizer in the four levels) was performed in a completely randomized design with four replications (each replication consists of three plants in a pot). Chl fluorescence parameters were measured in the three-factors factorial experiment (water stress and biofertilizer in the four levels and time in five levels) in the a completely randomized design with four replications. The data were statistically analyzed using *MSTATC* software (Michigan State University, USA) and the means were separated by *Duncan*'s multiple range test (P<0.05).

RESULTS

Fig. 1 and 2 show the maximum quantum yield of PSII photochemistry (F_v/F_m) of pistachio seedlings. Values of F_v/F_m below 0.800 were recorded for the plants of all treatments. Effect of biofertilizer and water stress treatments interaction, water stress and time interaction at the one percent level and biofertilizer and time interaction at the five percent level were significant for F_v/F_m (Table 1). F_v/F_m decreased with increasing water stress and lowest value of it was observed in severe water stress (25% FC) that in comparison with control (100% FC) was significantly different. In 25% FC treatment, F_v/F_m increased with using biofertilizer in comparison with control (100% FC) (Fig. 1). Different times had no effect on F_v/F_m at the control (100% FC) and mild water stress (75% FC), but in moderate water stress (50% FC) and severe water stress (25% FC), last time of measurement (fifth time) indicated the lowest F_v/F_m (Fig. 2a). At all levels of biofertilizer and also control, the lowest value of F_v/F_m was related to fifth time of measurement (Fig. 2b).

Table 1. *ANOVA* results for leaf area (La), chlorophyll fluorescence and pigments content of pistachio seedlings (*Pistacia vera* cv. Qazvini) exposed to varying intensities of water stress (S) and biofertilizer (B) treatments. T: time in F_v/F_m, F_v/F_m: maximal quantum yield of PSII photochemistry, Chl *a*: chlorophyll *a*, Chl *b*: chlorophyll *b*, Chl (*a*+*b*): total chlorophyll, Car: carotenoids

****: significant (P<0.001); **: significant (P<0.01); *: significant (P<0.05); ns: not significant.*

Parameters	S	B	S×B	T	S×T	B×T	S×B×T
La	5894.18^{***}	2287.43^{*}	1982.55^{**}				
F_v/F_m	0.011^{***}	0.004^{*}	0.003^{**}	0.057^{***}	0.018^{***}	0.002^{*}	0.002^{ns}
Chl *a*	0.05^{*}	0.03^{ns}	0.02^{ns}				
Chl *b*	0.07^{ns}	0.05^{ns}	0.12^{**}				
Chl (*a*+*b*)	0.5^{*}	0.3^{ns}	0.4^{*}				
Car	0.05^{*}	0.04^{*}	0.03^{*}				

Table 2. Correlation coefficients analysis in pistachio seedlings between leaf area (La), leaf pigments and chlorophyll fluorescence. F_v/F_m: maximal quantum yield of PSII photochemistry, Chl *a*: chlorophyll *a*, Chl *b*: chlorophyll *b*, Chl (*a*+*b*): total chlorophyll, Car: carotenoids

****: P<0.001; **: P<0.01; *: P <0.05; ns: not significant*

	Car	Chl (*a*+*b*)	Chl *b*	Chl *a*	F_v/F_m
La	-0.205^{ns}	-0.278^{*}	-0.276^{*}	-0.261^{*}	-0.047^{ns}
F_v/F_m	0.104^{ns}	0.077^{ns}	0.051^{ns}	0.079^{ns}	
Chl *a*	0.814^{***}	0.870^{***}	0.718^{***}		
Chl *b*	0.837^{***}	0.898^{***}			
Chl (*a*+*b*)	0.930^{***}				
Car					

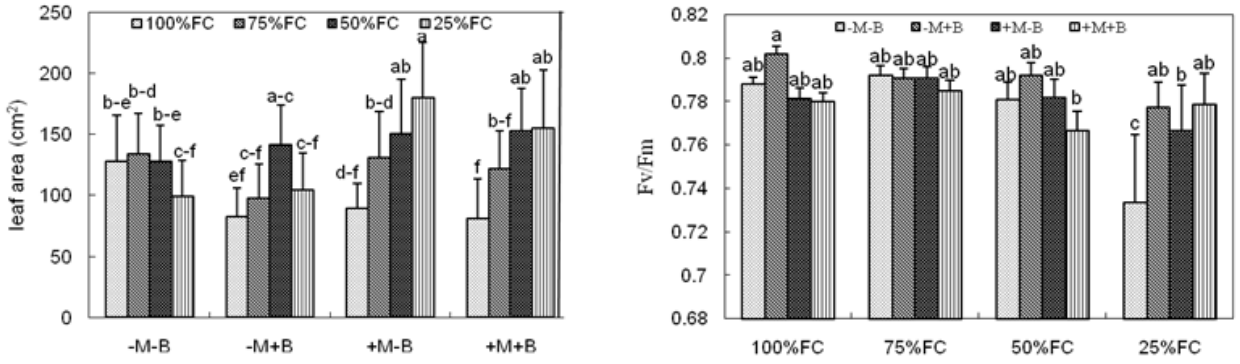

Fig. 1. Effect interaction of different water stress levels and biofertilizer on F_v/F_m in pistachio seedlings (*Pistacia vera* cv. Qazvini).Values are means and the vertical bars indicate standard error. -M-B: without mycorrhizae and bacteria, -M+B: *P. fluorescens* P_{52}, +M-B: *G. mosseae*,+M+B: *G. mosseae* + *P. fluorescens* P_{52}

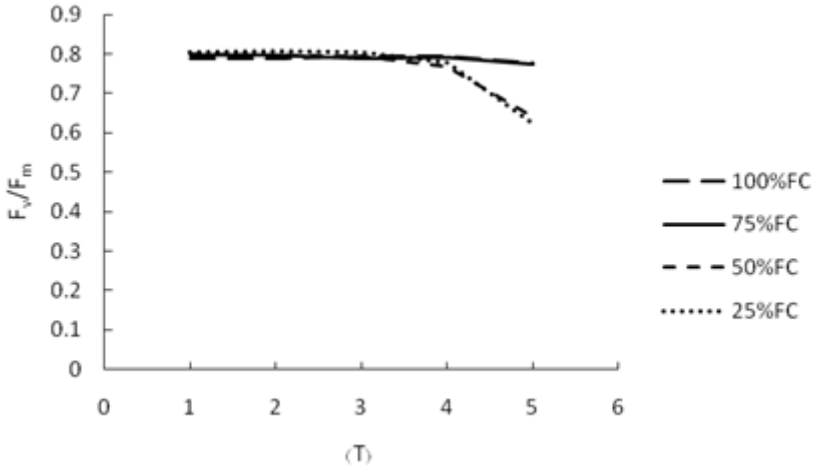

Fig. 2. Effect interaction of different water stress levels and time (a) and interaction of biofertilizer and time (b) on F_v/F_m in pistachio seedlings (*Pistacia vera* cv. Qazvini).Values are means. -M-B: without mycorrhizae and bacteria, -M+B: *P. fluorescens* P_{52}, +M-B: *G. mosseae*,+M+B: *G. mosseae* + *P. fluorescens* P_{52}. T_1: 20, T_2:34, T_3:50, T_4:65 and T_5:80 days after starting the water stress

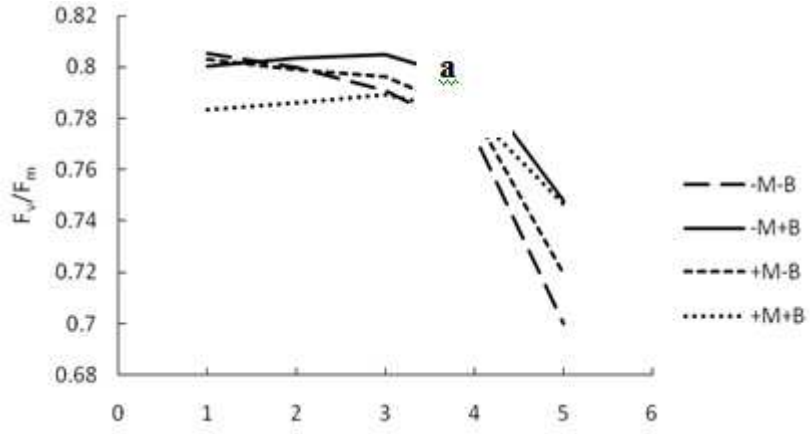

Fig 3. Effect interaction of different water stress levels and biofertilizer on leaf area in pistachio seedlings (*Pistacia vera* cv. Qazvini).Values are means and the vertical bars indicate standarderror. -M-B: without mycorrhizae and bacteria, -M+B: *P. fluorescens* P_{52}, +M-B: *G. mosseae*,+M+B: *G. mosseae* + *P. fluorescens* P_{52}

The results of the interaction between water stress treatments and biofertilizer levels on pistachio seedlings leaf area are reported in Fig. 3. In mycorrhizae treatment and combination treatment with bacteria and mycorrhizae, water stress from 25% and 50% FC caused significant increase in leaf area in comparison with control (100% FC). This increase in bacteria treatment was significant in comparison with control (100% FC) only in 50%FC.

According to Table 1, the treatment effect of water stress on Chl *a* was significant at the five percent level. As the results showed at the levels of 100% and 75% FC, the Chl *a* content was increased and in water stress from 25% and

50% FC showed a significant decrease in comparison with control (100% FC) (Fig. 4a). Changes in Chl *b* and total Chl content can be studied together. At the control (100% FC) Chl *b* and total Chl content in mycorrhizae treatment were significantly increased in comparison with the combination treatment with bacteria and mycorrhizae. At the severe water stress (25% FC), content of Chl *b* and total Chl in the combination treatment with bacteria and mycorrhizae were significantly decreased in comparison with control (Fig. 4b,4c). 25% FC treatment significantly reduced carotenoids content in the combination treatment with bacteria and mycorrhizae and mycorrhizae treatment (Fig. 4d).

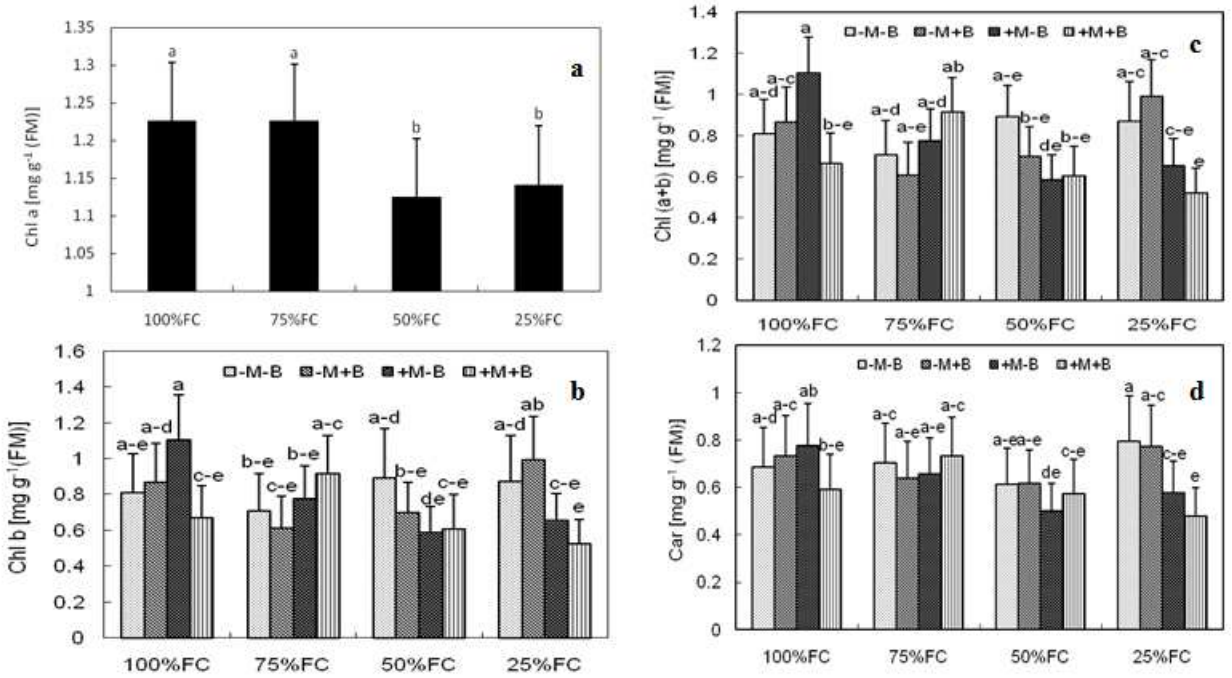

Fig. 4. Effect different water stress levels on chlorophyll (Chl) *a* (a) and interaction of different water stress levels and biofertilizer on Chl *b* (b), Chl (*a*+*b*) (c) and Car content (d) [mg g^{-1}(FM)] in pistachio seedlings (*Pistacia vera* cv. Qazvini).Values are means and the vertical bars indicate standarderror. -M-B: without mycorrhizae and bacteria, -M+B: *P. fluorescens* P_{52}, +M-B: *G. mosseae*,+M+B: *G. mosseae* + *P. fluorescens* P_{52}

DISCUSSION

The decrease in F_v/F_m is a good indicator of photoinhibitory damage caused by light when plants are subjected to environmental stresses [29]. In this experiment F_v/F_m decreased with increasing the water stress. These results are in agreement with the results of Kauser et al. [12], Oyetunji et al. [20], Ramires et al. [24], Szollosi et al. [33], Sayar et al. [29], Babaeian et al. [3] and Bagheri et al. [4]. Water stress reduces the quantum yield of PSII photochemistry that effect of it is reflected in the F_v/F_m ratio [20]. This suggests that electron transport from PSII to PSI is affected by water deficit. In plant species, Chl fluorescence indicates membrane integrity of thyllakoid and the relative efficiency of electron transport from PSII to PSI. The photosynthesis inhibition and Chl fluorescence increase can not only be due to the closing of stomata and Chl degradation, they can be a result of changes in the function of the thylakoid membranes and decrease in photochemical yield of the PSII for energy distribution between PSII and PSI [29].

According to the results of experiments, quantum yield of PSII photochemistry (F_v/F_m) increases by mycorrhizae fungi and despite destruction of the PSII reaction center of plant, growth increases in the presence of mycorrhizae [29]. The effects of the AMF are observed under water stress. The implication is that AMF can alleviate the adverse effect of water stress on quantum yield of PSII photochemistry. This indicates that AMF inoculation can enhance drought tolerance by ameliorating to some degree the injury done to the photosystems reaction centers [29]. Generally, AMF plays a role in F_v/F_m increase by improving plants nutritional status and activating mediated genes [19].

PGPR can improve plant growth by removing of pathogenic microorganisms, dissolving of insoluble phosphate and production of plant growth regulators [15]. Thus, F_v/F_m increases by improving the nutritional status of the plant, especially the available phosphorus (P) element for plant photosynthesis and also improving photosynthesis and plant growth under water stress. In this regard, the results of the present experiment are in agreement with the work of Gamalero et al., [9] and F_v/F_m was increased by application of biofertilizer.

At the present experiment, in the time and water stress interaction, F_v/F_m decreased with time and the lowest F_v/F_m was observed in the fifth time. This reduction was significant only in the moderate and severe water stress. The duration and level of stress is very important in the living stresses. Plants are affected by water stress with increasing duration and level of water stress. Chl fluorescence is a well parameter to show this subject. Also at all levels of biofertilizer, F_v/F_m decreased with time.

In mycorrhizae treatment and combination treatment with bacteria and mycorrhizae, water stress from 25% and 50% FC caused significant increase in leaf area in comparison with control (Fig. 3). These results are in agreement with the results of Ravnskov and Jakobsen [25] and Sabannavar and Lakshman [26]. Pseudomonas fluorescent bacteria are important in terms of production of plant growth regulators, particularly auxin [22] (auxin increases plant growth by elongation of plant cells, stimulate of cell division and differentiation of plant cells), iron chelating compounds, organic acids (succinic acid and lactic) and solving phosphorus by secretion of organic acids and phosphatase [14] and increase plant growth [11]. In the water stress condition, mycorrhizae increases macro (especially phosphorus) and micro nutrient uptake than plants without mycorrhizae. As a result, mycorrhizae increases tolerance to water stress by improving plants nutritional status [19]. Thus, increase of phosphorus uptake by mycorrhizae considering the role of phosphorus in molecular structures such as nucleic acids, stomatal conductance and photosynthesis increases the tolerance of plants against water stress [28].

At this experiment, the amount of Chl *a* decreased with increasing water stress. Chloroplast and plant pigments are usually affected by water stress. For example, water stress reduces Chl *a* and *b* by hydrolysis of thylakoid proteins [34]. Woodward and Bennett [36] reported that decreasing water in the cell reduces concentration of Chl *a* and *b*. They stated that the reduction of Chl concentration is due to chlorophyllase enzyme activity. Bagheri et al. [4] showed that in the pistachio seedlings, concentration of Chl *a*, *b* and total decreased with increasing water stress. Car content decreased with increasing water stress, too. The results of this experiment are consistent with the results of the several experiments at this regard [6, 18, 17, and 21]. In the present experiment, application of biofertilizer increased leaf area with increasing water stress. Therefore, due to the increase in leaf area, pigments can be reduced due to their dilution [21]. A field experiment was done on wheat and showed that decrease in plants Car content in water stress may be dependent on changes in leaf and chloroplast membrane domain [17]. Under severe water stress, all pigments reduced in the combination treatment with bacteria and mycorrhizae. There is a negative correlation between pigments and leaf area that is shown in the Table 2. As a result, reducing pigments in this level can be dependent on the dilution effect.

CONCLUSION

It could be concluded from this study that application of AMF and PGPR enhance tolerance of plants to water stress with increasing F_v/F_m. Maintain the efficiency of the photosynthetic apparatus is a feature of drought tolerance plants. The results of this study showed that the pistachio plant is tolerance to water stress, because reduce the efficiency of the photosynthetic apparatus occurred in water stress 25% FC and in the last time of measurement.

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