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Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) modulate physiological mechanisms to tolerate drought stress due to arbuscular mycorrhizal fungi and mycorrhizal helper bacteria

Prananath Barman[1]*, Sanjay Kumar Singh[2], Vishwa Bandhu Patel[3], Lata Nain[4] and Avnish Pandey[5]

[1]Division of Crop Production, Central Institute for Subtropical Horticulture, Rehmankhera, P.O. Kakori, Lucknow, Uttar Pradesh, 227 107 India.
[2]Division of Fruit Science and Horticulture Technology, Indian Agricultural Research Institute, New Delhi, 110 012 India.
[3]Division of Fruit Science, Bihar Agricultural University, Sabour, Bihar, 813 210 India.
[4]Division of Microbiology, Indian Agricultural Research Institute, New Delhi, 110 012 India.
[5]Division of Crop Improvement, Central Institute for Subtropical Horticulture, Rehmankhera, P.O. Kakori, Lucknow, Uttar Pradesh, 227 107 India.

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The influence of *Glomus intraradices* colonisation on growth and reactive oxygen metabolism of Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) seedlings treated with mycorrhizal helper bacteria, that is, phosphate solubilising bacteria containing a mixture of *Bacilus subtilis* and *B. megaterium*, *Azospirillum braisilence* or *Providencia* sp.) in potted culture was studied under well-watered or drought stress conditions. Results indicate that mycorrhizal inoculation increased plant growth and nutrient acquisition independent of the water regime, particularly when associated with mycorrhizal helper bacteria. Exposure of plant to drought stress led to generation of superoxide radicals and hydrogen peroxide in leaf tissues, however, their concentrations were lower in seedlings inoculated with *G. intraradices* and phosphate solubilising bacteria, as compared to other treatments including control. This particular treatment also increased total glutathione content and enhanced antioxidant enzyme activity in plant and microbial activity in soil. Mycorrhizal colonization was positively correlated with antioxidant metabolite, plant antioxidant enzymes, rhizospheric microbial activity and microbial biomass carbon and negatively correlated with reactive oxygen species under drought stress, which indicated that its inoculation could enhance plant defence system and alleviates oxidative damages to membrane lipids and proteins.

Key words: Cleopatra mandarin, superoxide radicals, hydrogen peroxide, anti-scavenging enzymes, plant nutrients, soil microbial biomass, soil microbial activity.

INTRODUCTION

Drought stress is a major abiotic factor that limits plant's growth, thus becoming one of the growing concerns in agriculture management around the world (Saeidnejad et al., 2013). The mycorrhizal association with roots of most

of the plants not only stimulate the plant growth, but also contribute in enhancing tolerance to drought (Navarro et al., 2011).

The free living and endo-symbiotic bacteria, known as “mycorhhizal helper bacteria” (MHB) stimulate pre-symbiotic fungal growth leading to an increase in root-fungus contacts and root colonization (Frey-Klett et al., 2007). The inoculation of *Glomus intraradices* along with PSB or *Azospirillum brasilense* can improve plant growth than single inoculation (Toro et al., 1997; Ruiz-Sanchez et al., 2011). *Providencia* sp., the bacterial strain AW5 (accession no FJ866760), used in the present investigation, was shown to have potential to stimulate plant growth through phosphorus solubilization, ammonia and indole-3-acetic acid production (Rana et al., 2011a).

Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) produces good quality fruits in scion variety for most of the citrus species (Ouko and Abubaker, 1988). The potential benefit of *G. intraradices* has been reported in Cleopatra mandarin (Camprubi et al., 1995). However, no work has been carried out to study the physiology of citrus plant under drought stress, when co-inoculated with AMF and MHB. Hence, *G. intraradices* alone or with MHB was used in the study so as to determine the suitable microbial combination for improving adaptive behaviour of Cleopatra mandarin against drought stress.

MATERIALS AND METHODS

Site of experimentation

The potted culture experiment was conducted in glasshouse at Indian Agricultural Research Institute (IARI), New Delhi, located at 77°12' E longitude, 28°40' N latitude and an altitude of 228.6 m above mean sea level. Climate is categorized as semi-arid, subtropical with hot dry summer and cold winter.

Source of microbial inoculants

The AMF *G. intraradices* was procured from Division of Agricultural Microbiology, Kittur Rani Channamma College of Horticulture, Arabhavi, Karnataka. The starter culture was multiplied in ragi (*Elevusine coracana*), raised in plastic pot (12 × 20 cm) filled with a mixture of soil, sand and farm-yard manure (FYM) (2:2:1) that had been autoclaved (1.05 kg cm^{-2}) for 2 h. The inoculum was sealed in a polythene packet consisting of freshly collected rhizosphere soil and AMF spores along with hyphae, arbuscules, vesicles and root segments of ragi plant.

Providencia sp. (AW5) was isolated from the wheat rhizosphere and identified using 16S rDNA sequence analysis in the division of Microbiology, IARI and submitted to NCBI (Accession No. FJ866760) (Rana et al., 2011b). Inoculum of bacterial strain was prepared by growing in nutrient broth at 28 ± 1°C for 48 h at 100 rpm, such that the inoculum contained 10^{11} cells ml^{-1}.

The other bacteria like *A. brasilense* and PSB (*Bacilus subtilis* + *Bacilus megaterium*) were cultured in nutrient broth and then multiplied in carrier like finely powdered and sterilized charcoal powder. The broth containing 10^{9} cells ml^{-1} was added to 1/3 of the water holding capacity of the carrier.

Plant material, microbial inoculation and growth conditions

The seeds of Cleopatra mandarin were collected from the germplasm of Division of Fruits and Horticultural Technology, IARI and then surface sterilized by immersing in 70% alcohol for 5 min, followed by rinsing three times with sterile distilled water and then kept over wet filter paper in Petri dishes at 28°C for germination. After 7 days, the seedlings were planted in plastic containers (12 × 20 cm) containing 4.1 kg of mixture of sterilised soil : sand : FYM (2:2:1) having $EC_{(1:2)}$ 6.35 mS m^{-1}, $pH_{(1:2)}$ 7.92, HCO_3^- 1.14 g kg^{-1} and Cl^- 5910.75 ppm.

During planting, the seedlings were inoculated with either AMF (5 g per kg of potting mixture consisting of 80 to 88 infective propagules per 5 g of inoculum) or bacterial species (5 g per kg of potting mixture containing 10^{9} cells per g of carrier) or both. In case of *Providencia* sp. (AW 5), 5 ml of liquid media containing 10^{11} cells per ml of media was used for each pot. The seedlings were maintained in glasshouse with day-night temperatures of 27 ± 1°C and humidity of 80-85%.

Day lengths were extended up to 16 h with cool white fluorescent lights at 630 µmol m^{-2} s^{-1} for improving the vegetative growth of the plants. Seedlings were watered on alternate days with 250 ml of autoclaved water, maintaining soil moisture content above 80% using soil moisture meter. Water used for irrigation in high density orchard had $EC_{(1:2)}$ 288 µS m^{-1}, $pH_{(1:2)}$ 7.48, HCO_3^- 1.0 milliequivalent/litre and Cl^- 110.76 ppm.

Imposition of differential moisture regimes

The differential water treatments were started at 270 DAI under glasshouse condition. Half of seedlings under each treatment received ample water (750 ml) at an interval of two days and the remaining half were imposed drought stress by withholding water. Daily soil relative water content was measured using soil moisture meter (FieldScout TDR 300, Spectrum Technologies, Inc.) fitted with 4.8-inch probe rods. Wet point was fixed at 90% and dry point at 8%. The soil relative water content (RWC) for well-watered (WW) seedlings was monitored at 80%. Entire plants were harvested after 20 days, when drought stressed (WS) seedlings showed visible symptoms of temporary wilting.

Measurements

Root colonization

Per cent root colonization was determined by using the method as detailed by Phillips and Haymann (1970). The presence of fungal hyphae, arbuscles and vesicles were observed under 10x

*Corresponding author. E-mail: Prananath.india@gmail.com. Tel: +91-0522-2841022. Fax: +91-0522-2841025.

Abbreviations: AMF, Arbuscular mycorrhizal fungi; **MHB,** mycorrhizal helper bacteria; **PSB,** phosphate solubilising bacteria; **WS,** water stress; **WW,** well-watered; **DAI,** days after inoculation.

microscope (Nickon TS 100).

Fresh and dry weight of shoot and root

Fresh weight of root and shoot of each plant was recorded by electronic balance. The samples were then put in the perforated paper bag and kept in hot air oven at 70°C until constant dry weight.

Leaf nutrient analysis

For tissue nutrient analyses, oven-dried samples were ground, sieved and digested in nitric acid : perchloric acid (9:4). Total nitrogen (N) was determined in samples of 0.5 g dry weight using the Kjeldahl method. Phosphorous (P) was analysed by a vanadatemolybdate method. The P transmittance was read at 420 nm. Potassium (K) was determined with the help of flame photometer (Systronics 128, Ahmedabad) using specific filter and LPG flame. Determination of other foliar nutrients like Ca, Mg, Zn, Fe, Cu and Mn was done by the atomic absorption spectrophotometer (GBC-Avanta PM; GBC-Advanta Scientific Equipment, Dandenong, Victoria, Australia) using nitrous oxide-acetylene flame.

Reactive oxygen species

Leaf samples (1 g) were homogenised in 5 ml of pre-cooled phosphate buffer (0.2 M, pH 7.2) containing 1 mM diethyl dithiocarbamate and centrifuged at 5000 g for 5 min. Superoxide radical was estimated as per the method of Chaitanya and Naithani (1994). Hydrogen peroxide was estimated as per the method of Rao et al. (1996).

Antioxidant enzymes

Aliquots (1 g) of roots were homogenized in 5 ml of chilled phosphate buffer (0.1 mol l^{-1}, pH 7.8) and centrifuged at 15,000 g for 20 min at 4°C (Hermle Z 323K), and the supernatant was used for enzyme assays. The activity of catalase (CAT, EC 1.11.1.6) was estimated by titrating the reaction mixture containing 1 ml of enzyme extract and 2 ml of H_2O_2 against $KMnO_4$ (0.01 M) till the appearance of faint pink colour persisting at least for 15 s (Luck, 1974). The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured as a decrease in absorbance at 290 nm as ascorbate was oxidised by H_2O_2 (Nakano and Asada, 1981). Guaiacol-peroxidase (G-POD, EC 1.11.1.7) activity was measured by following the change of absorption at 560 nm due to guaiacol oxidation (Thomas et al., 1981). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Beyer and Fridovich (1987). Glutathione reductase (GR, EC 1.8.1.7) assay was determined based on the formation of reduced glutathione with 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), which absorbs at 412 nm, as proposed by Carlberg and Mannervik (1985). Protein concentrations were determined by a modified Lowry method (Hartree, 1972) with bovine serum albumin as a standard protein.

Non-enzymatic antioxidant

Total glutathione (GSH + GSSG), an important antioxidant, was determined according to the method of Grifith (1985) and Smith (1985).

Soil microbiological parameters

Alkaline phosphatase activity was assayed as per the method of Tabatabai and Bremner (1969) and the enzymatic activity was expressed as µg of ρ-nitrophenol g^{-1} soil dry weight h^{-1}. Dehydrogenase activity, expressed as µg of triphenyl formazon g^{-1} soil dry weight day^{-1}, was assayed as per the method of Casida et al. (1964). Microbial biomass carbon (MBC), expressed as µg g^{-1} soil sample, was estimated by the method of Nunan et al. (1998).

Experimental design and statistical analysis

Experiments were laid out in a completely randomised block design with eight treatments and three replications per treatment. The treatment details are as follows: T_1 = Control; T_2 = PSB alone; T_3 = *A. brasilense* alone; T_4 = *Providencia* sp. (AW5) alone; T_5 = *G. intraradices* alone; T_6 = *G. intraradices* and PSB; T_7 = *G. intraradices* and *A. brasilense*; T_8 = *G. intraradices* and *Providencia* sp. (AW 5).

Experimental data from a period of two years was pooled and then subjected to analysis of variance (ANOVA) using statistical analysis software SPSS package (SPSS 11.0) and means were evaluated by Fisher's protected least significant difference (LSD). Differences at $P < 0.05$ were considered significant.

RESULTS

Drought stress had a strong effect on AMF development. The present study revealed significantly highest root colonisation in seedlings co-inoculated with *G. intraradices* and PSB under WS and WW conditions (62.50 and 83.33%, respectively). Colonization was not observed in the roots of non-AM seedlings under WS (Figure 1A).

Shoot and root weight was reduced under drought stress, irrespective of the treatment provided (Table 1). However, co-inoculation of *G. intraradices* and PSB increased respectively fresh and dry weight of shoot (281.60 and 377.94%) and that of root (547.98 and 578.18%), as compared to control. Under WW condition, fresh and dry weight of shoot was increased respectively by 221.57 and 213.66% and that of root by 309.34 and 367.35%, respectively, in seedlings co-inoculated with *G. intraradices* and PSB.

The super oxide radical (O_2^-) in leaf was found to be produced more under WS condition, as compared to WW condition, regardless of any treatment (Figure 1B). However, production was significantly lesser by 27.69 and 28.80% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control, under WS and WW conditions, respectively, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) under WS condition (25.81 and 24.73%, respectively) and *G. intraradices* and *Providencia* sp. (AW 5) under WW condition (24.61%).

The accumulation of hydrogen peroxide (H_2O_2) was reduced by 21.97% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control (Figure

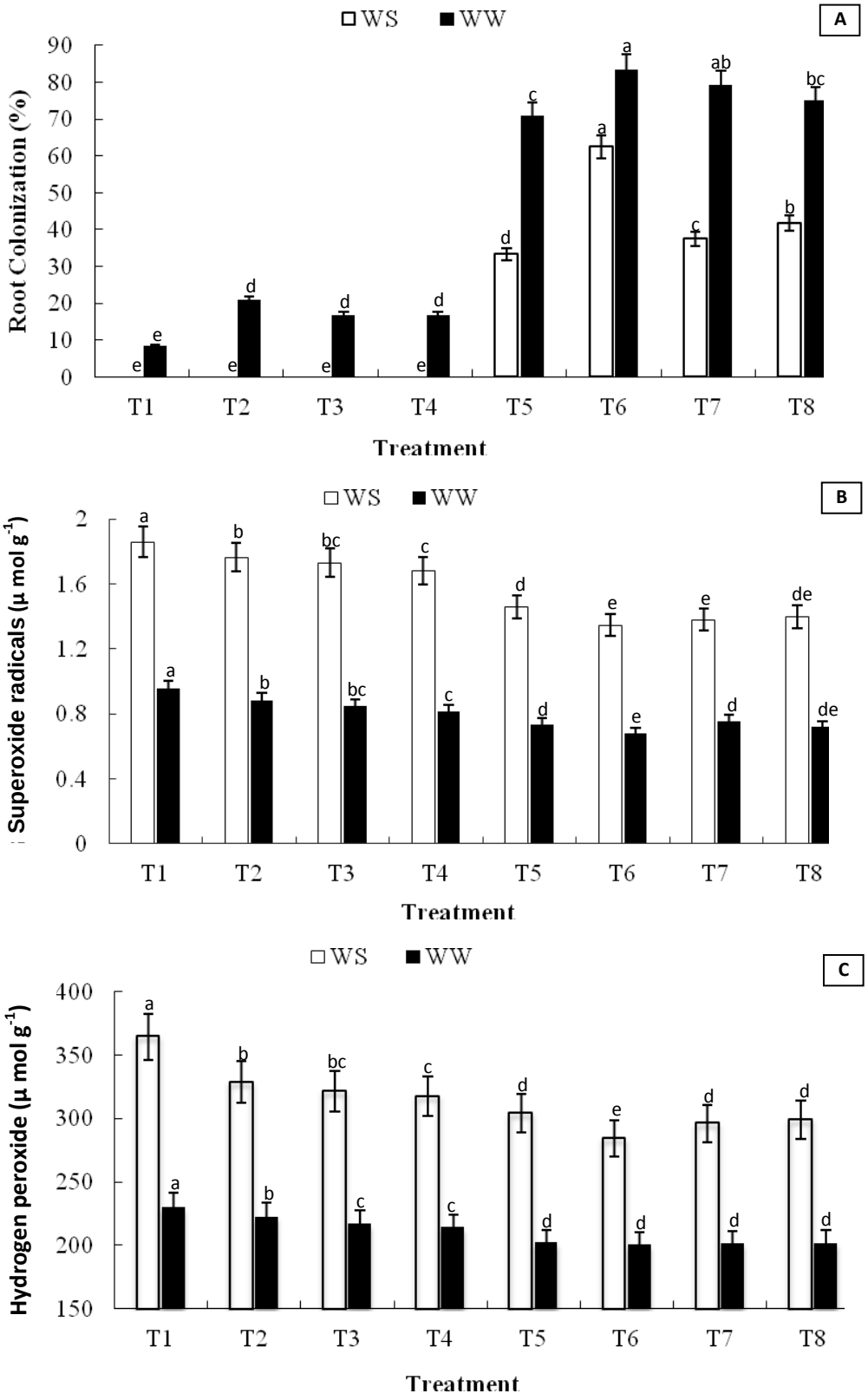

Figure 1. Influence of microbial inoculants on (A) AMF colonization in root (12 root segments observed per treatment); (B) Superoxide radicals; (C) hydrogen peroxide content in fresh leaves of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at $p \leq 0.05$, $n = 2$ (Web Agri Stat Package 2.0).

Table 1. Response of Cleopatra mandarin to microbial inoculants on shoot and root weight (g plant^{-1}).

T	Fresh shoot weight		Fresh root weight		Dry shoot weight		Dry root weight	
	WS	WW	WS	WW	WS	WW	WS	WW
T_1	5.11±0.84f	11.15±0.32f	2.59±0.57e	7.55±0.08f	2.68±0.37f	6.26±0.32e	1.54±0.30f	3.40±0.22e
T_2	8.84±0.50e	13.96±0.57e	5.90±0.86d	7.56±0.50f	4.77±0.50e	6.91±0.57de	1.93±0.42ef	3.68±0.21e
T_3	11.70±0.06d	14.34±0.52e	9.62±0.11c	10.52±0.01e	6.69±0.06d	8.79±0.23c	3.31±0.39d	6.06±0.04cd
T_4	11.09±0.10d	13.63 ±0.57e	8.45±0.24c	9.54±0.23e	5.46±0.10e	7.88±0.28cd	2.85±0.05de	5.09±0.03d
T_5	12.11±0.26d	20.32± 0.75d	10.10±0.06c	11.82±0.04d	7.29±0.03d	12.74±0.32b	4.67±0.15c	7.10±0.25c
T_6	19.51±0.32a	35.86±0.81a	16.79±0.51a	30.89±0.47a	12.83±0.32a	19.64±0.52a	10.41±0.51a	15.89±0.53a
T_7	16.65±0.64b	26.82±0.83b	13.34±0.75b	19.82±0.49b	10.61±0.64b	13.50±0.81b	6.63±0.23b	11.18±0.72b
T_8	14.28±0.18c	23.38±0.81c	12.42±0.87b	16.74±0.44c	8.59±0.12c	13.46±0.29b	5.94±0.06b	10.36±0.72b

Mean ± standard error, n = 2; values followed by same letter in a column are not significantly different ($p \leq 0.05$).

1C). Under WW condition, H_2O_2 content was significantly lesser in seedlings co-inoculated with *G. intraradices* and PSB, *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) and *G. intraradices* alone (12.83, 12.30, 12.21 and 12.08%, respectively), as compared to the control.

Drought stress had positive effect on increase in activities of leaf enzymes in Cleopatra mandarin (Figure 2A, B, C, 3A and 3B). The co-inoculation of *G. intraradices* and PSB enhanced the activities of CAT (53.60%), APX (46.85%), G-POD (27.77%) and GR (73.25%), which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* for CAT and G-POD (51.36 and 26.39, respectively) and *G. intraradices* and *A. brasilense*, *G. intraradices* and *Providencia* sp. (AW 5) and *G. intraradices* alone for APX (42.31, 41.43 and 38.13, respectively), whereas *G. intraradices* in combination with *A. brasilense* or PSB exhibited increased SOD activity by 74.01 and 71.60%, respectively, as compared to control under WS condition. Under WW condition, activities of CAT, APX, G-POD, SOD and GR were respectively increased by 10.58, 17.55, 22.64, 141.70 and 114.89% in seedlings co-inoculated with *G. intraradices* and PSB, as compared to control, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* and single inoculation of *G. intraradices* for CAT (9.19 and 9.05%, respectively) and co-inoculation of *G. intraradices* and *A. brasilense* for APX (16.64%).

The co-inoculation of *G. intraradices* and PSB significantly stimulated highest increase in total glutathione in leaf under WS and WW conditions (24.47 and 27.82%, respectively), as compared to control, which was statistically at par with co-inoculation of *G. intraradices* and *A. brasilense* (22.24%) under WS (Figure 3C).

The leaf nutrient content was found to be influenced by microbial inoculation, regardless of any moisture regime. The leaf N content was 58.19 and 38.13% higher in seedlings co-inoculated with *G. intraradices* and *A. brasilense* under WS and WW, respectively, as compared to the control, which was statistically at par with seedlings inoculated with *Azospirillum* alone (34.92%) under WS (Table 2). The synergistic effect of *G. intraradices* and PSB resulted in significant increase in leaf P, K, Ca and Mg content, regardless of any moisture regime, as compared to the control, which was statistically at par with inoculation of *G. intraradices* alone and *Azospirillum brasilense* alone under WS and WW, respectively, for Ca content, inoculation of *G. intraradices* alone and co-inoculation of *G. intraradices* and *A. brasilense* under WW condition for Mg content (Table 2).

The drought stress also reduced leaf micronutrient content. However, inoculation of *G. intraradices* along with PSB resulted in significantly higher level of leaf Fe, Cu, Mn and Zn content, regardless of any moisture regime, which was statistically at par with co-inoculation of *G. intraradices* and *Providencia* sp. (AW 5) under WS for Cu content, co-inoculation of *G. intraradices* and *A. brasilense* under WS condition for both Mn and Zn content (Table 3).

The analysis of rhizospheric soil inoculated with different microbial culture including control revealed the superiority of co-inoculation of AMF and MHB over single inoculation and in particular, co-inoculation of *G. intraradices* and PSB for alkaline phosphatase activity under WS and WW conditions (81.02 and 87.30%, respectively), as compared to the control (Figure 4A).

Drought stress had negative effect on soil dehydrogenase activity (Figure 4B). However, it had significantly lesser effect in the rhizosphere of seedlings inoculated with *G. intraradices* and PSB (61.55%) as compared to the control. The particular treatment also showed 49.61% more enzyme activity, as compared to the control, under WW condition.

Microbial biomass carbon (MBC) was affected by different microbial treatment, however, inoculation of AMF and MHB had significant effect than single inoculation or uninoculated control (Figure 4C). Under WS and WW conditions, the MBC content was 192.73 and 100.04% more in *G. intraradices* + PSB inoculated rhizosphere as compared to the control, which was

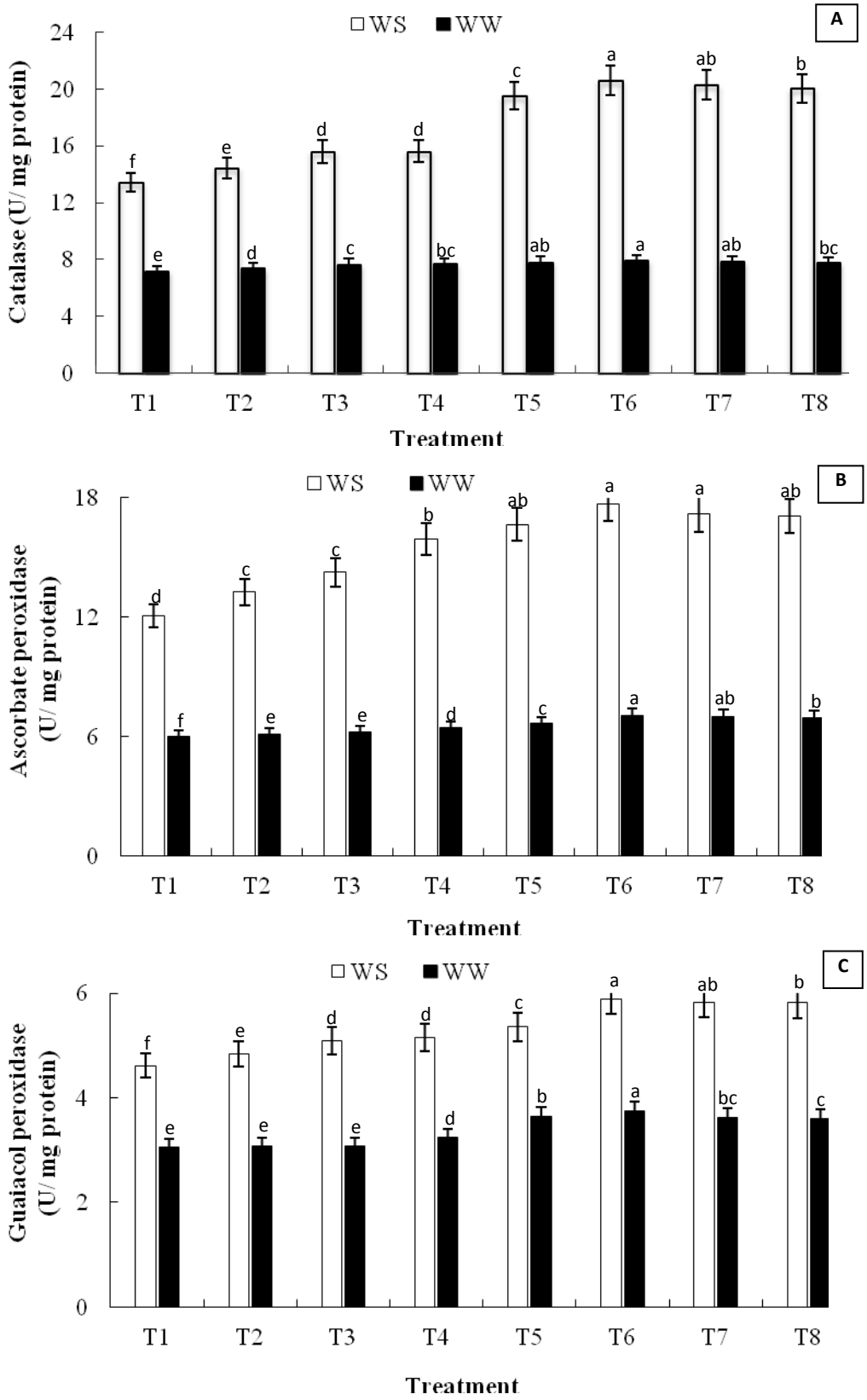

Figure 2. Influence of microbial inoculants on (A) Catalase; (B) Ascorbate peroxidise; (C) Guaiacol peroxidase content in fresh leaves of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at $p \leq 0.05$, n = 2 (Web Agri Stat Package 2.0).

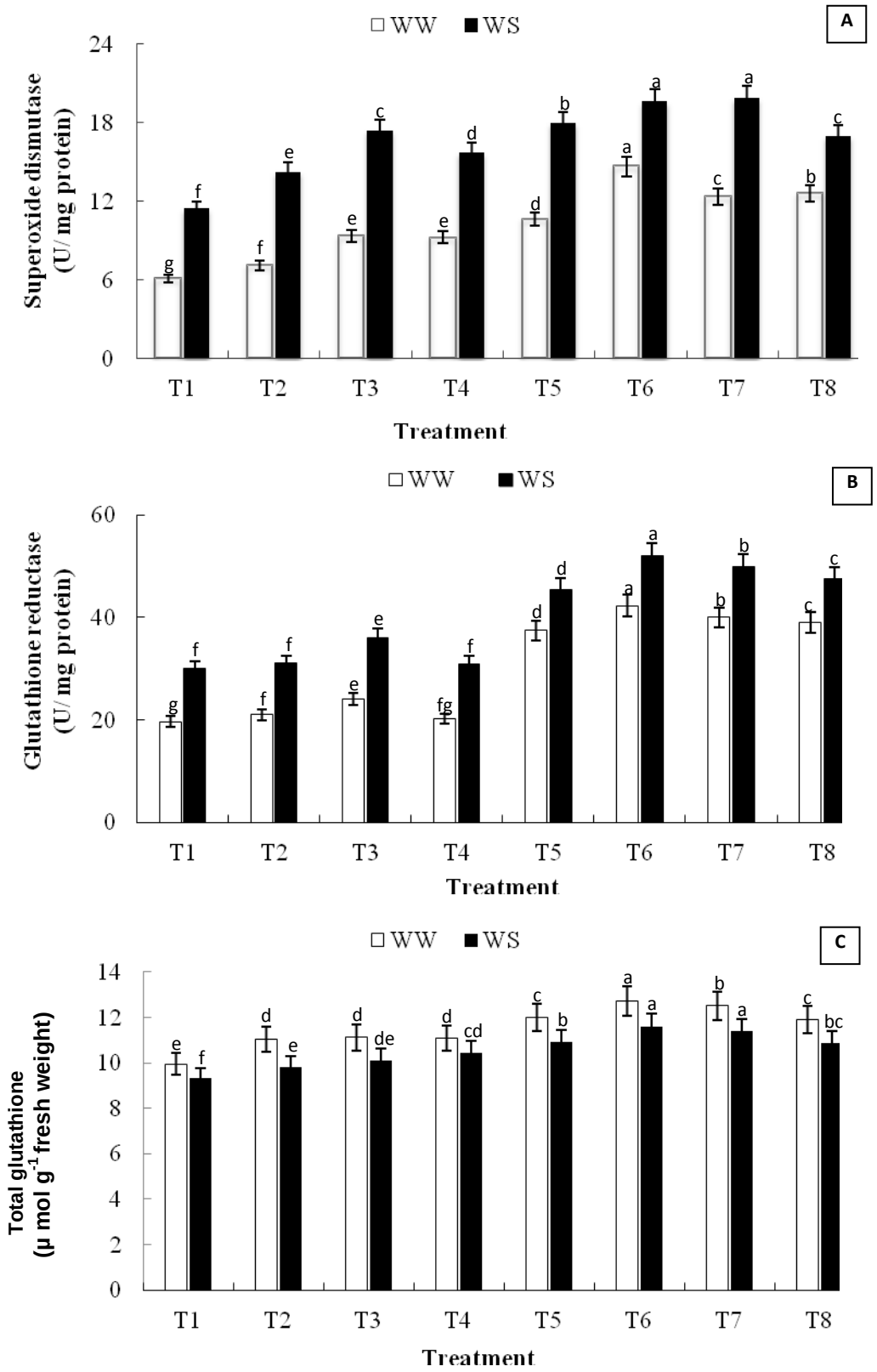

Figure 3. Influence of microbial inoculants on (A) superoxide dismutase; (B) glutathione reductase; (C) total glutathione content in fresh leaves of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at $p \leq 0.05$, n = 2 (Web Agri Stat Package 2.0).

Table 2. Response of Cleopatra mandarin to microbial inoculants on leaf macro nutrient content (%).

	N		P		K		Ca		Mg	
	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW
T_1	1.16±0.03f	1.48±0.08d	0.18±0.01e	0.25±0.02e	1.40±0.01f	1.80±0.01g	1.48±0.03e	1.28±0.03f	0.10±0.01d	0.18±0.02c
T_2	1.36±0.04e	1.70±0.01c	0.24±0.02d	0.27±0.01de	1.65±0.02d	2.04±0.01d	1.61±0.05d	1.44±0.07e	0.13±0.01c	0.20±0.02c
T_3	1.76±0.01b	1.99±0.00a	0.25±0.02d	0.27±0.01de	1.62±0.01e	2.01±0.00ef	1.80±0.01b	1.75±0.02a	0.11±0.01cd	0.19±0.03c
T_4	1.43±0.02d	1.70±0.02c	0.28±0.02cd	0.29±0.01cd	1.59±0.01e	2.00±0.01f	1.68±0.02c	1.44±0.03e	0.12±0.00cd	0.20±0.01c
T_5	1.56±0.01c	1.77±0.00c	0.34±0.02b	0.33±0.01b	1.80±0.01b	2.09±0.01c	1.94±0.01a	1.53±0.01de	0.17±0.01b	0.27±0.01ab
T_6	1.74±0.03b	1.88±0.01b	0.43±0.01a	0.42±0.01a	1.87±0.01a	2.13±0.00a	1.99±0.00a	1.72±0.02ab	0.21±0.01a	0.31±0.00a
T_7	1.84±0.01a	2.04±0.01a	0.32±0.01bc	0.31±0.01bc	1.80±0.00b	2.11±0.00b	1.85±0.01b	1.65±0.02bc	0.18±0.00b	0.29±0.00ab
T_8	1.62±0.01c	1.86±0.02b	0.32±0.02bc	0.31±0.02bc	1.73±0.00c	2.02±0.01e	1.82±0.00b	1.61±0.01cd	0.17±0.01b	0.26±0.00b

Mean ± Standard error, n = 2; values followed by same letter in a column were not significantly different ($p \leq 0.05$).

Table 3. Response of Cleopatra mandarin to microbial inoculants on leaf micro nutrient content (ppm).

	Fe		Cu		Mn		Zn	
	WS	WW	WS	WW	WS	WW	WS	WW
T_1	26.93±0.55g	37.44±0.12g	6.98±0.06d	8.29±0.03e	70.64±0.43g	77.65±0.27f	8.45±0.58e	12.71±0.35d
T_2	37.56±0.07e	40.91±0.36f	8.05±0.03c	9.18±0.01d	78.18±0.95f	80.94±0.04e	11.99±1.58d	18.18±1.08bc
T_3	40.15±0.02d	47.02±0.21d	7.95±0.04c	9.17±0.03d	79.63±0.04e	81.28±0.01e	12.54±1.33d	17.59±0.91c
T_4	35.15±0.02f	42.08±0.27e	7.82±0.17c	8.98±0.05d	81.45±0.13d	83.05±0.04d	15.04±1.05bcd	19.03±1.16bc
T_5	46.51±0.18c	50.71±0.24b	9.44±0.20b	10.50±0.21c	87.56±0.25c	89.74±0.08c	17.25±1.71bc	20.48±1.30bc
T_6	49.96±0.18a	52.74±0.03a	10.14±0.01a	11.98±0.01a	90.22±0.19a	91.58±0.18a	21.09±0.13a	27.31±0.70a
T_7	47.46±0.12b	50.76±0.05b	9.66±0.12b	10.93±0.03b	89.15±0.01ab	90.88±0.06b	17.89±1.11ab	21.39±1.31b
T_8	46.64±0.03c	49.71±0.03c	10.30±0.30a	11.12±0.26b	88.68±0.06bc	90.84±0.11b	14.46±0.44cd	20.69±1.51bc

Mean ± Standard error, n = 2; values followed by same letter in a column were not significantly different ($p \leq 0.05$).

statistically at par with that of rhizosphere of seedlings co-inoculated with *G. intraradices* and *Providencia* sp. (AW 5) under WW condition (86.51%).

DISCUSSION

Colonization of roots by AMF and the subsequent benefits derived by a host plant depend initially on the survival of AMF propagules, particularly spores. The higher *G. intraradices* colonization in roots of Cleopatra mandarin treated with PSB might be due to improved AMF interaction with plant roots due to production of active metabolites such as vitamins, amino acids and indole-3-acetic acid by the bacteria (Vivas et al., 2003), resulting in increased germination of fungal spores and rapid AMF establishment in soil (Toljander et al., 2006).

Drought had adverse effect on biomass production in Cleopatra mandarin. The synergistic effect of PSB with *G. intraradices* for increase in both shoot and root weight indicated its compatible interaction with AMF. This increase in plant growth has been attributed to the stimulation of activity of AM fungal mycelium in the rhizosphere by PSB (Marulanda et al., 2003), resulting in improving root-fungus interaction, thereby increasing the hyphal efficiency for exploring greater volume of soil for water and nutrient uptake (Allen, 2011).

The AMF inoculation can lower the H_2O_2 accumulation in plants (Wu et al., 2006) and also enhance the production of SOD involved in catalyzing the conversion of free O_2^- to O_2 (Huang et al., 2010). Thus, lower ROS levels in leaves of Cleopatra mandarin co-inoculated with AMF and MHB might be due to the fact that bacteria induced a higher increase in antioxidant enzyme activities in AM plants in response to stress, resulting in alleviating the negative effect of stress (Kohler et al., 2009).

The AMF possess several special genes encoding for antioxidant enzymes, whose expression patterns can regulate the activities of antioxidant enzymes (Wu and Zou, 2009). In the present study, the enhanced antioxidant activity in AMF and MHB co-inoculated seedlings supports the view that increased enzyme activities could be involved in the beneficial effects of microbial inoculation on the performance of plants grown under semi-arid conditions (Alguacil et al., 2003), indicating better plant protection against the drought stress (Azcón et al., 2013).

The plants inoculated with AMF can accumulate antioxidants to counteract ROS under any environmental stress (Kaya et al., 2009). The synergistic effect of MHB

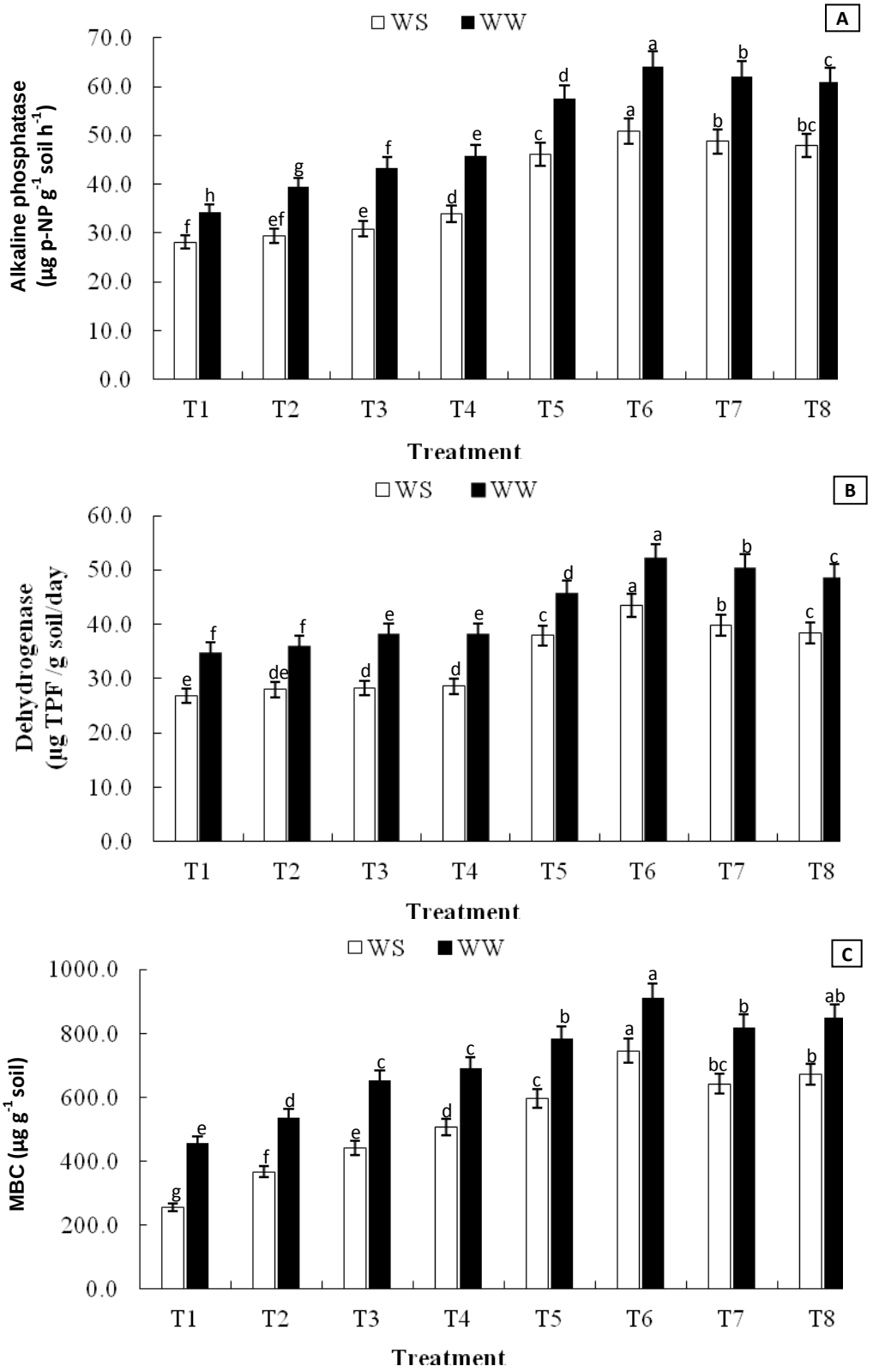

Figure 4. Influence of microbial inoculants on (A) alkaline phosphatase; (B) dehydrogenase; (C) microbial biomass carbon (MBC) in rhizosphere of Cleopatra mandarin grown in pots under differential moisture regime condition. WS, water stress; WW, well-watered. The bars of treatment in particular moisture regime having same letter do not differ significantly at $p \leq 0.05$, n = 2 (Web Agri Stat Package 2.0).

with AMF leading to higher antioxidant level might help the host plant in dissipating the photo-synthetically produced electrons and in alleviating oxidative damage.

The higher foliar N content in Cleopatra mandarin co-inoculated with *G. intraradices* and *A. brasilense* could be attributed to the increase in N-assimilating enzymes, such as nitrate reductase in the shoots of AMF colonised plants (Caravaca et al., 2005). The higher level of P in seedlings co-inoculated with *G. intraradices* and PSB might be due to release of phosphate ions from sparingly soluble inorganic and organic P compounds in soil, thereby contributing to increased soil phosphate pool available for the extraradical AM fungal hyphae to pass on to the plant (Artursson et al., 2006). The enhanced acquisition of other mineral nutrients (K, Ca, Mg, Fe, Cu, Mn and Zn), in co-inoculated microbial treatment, could be attributed to the greater absorption of the surface area provided by extensive fungal hyphae (Navarro et al., 2011). The acquisition of P and Ca by AM plants were recorded lesser under WW than WS condition, which could be attributed to improved efficiency of fungal hyphae in transfer of nutrients especially phosphorus and calcium which are immobile in soil and plant, respectively, from nutrient depletion zone to root cortex. Bryla and Duniway (1997) reported that AM fungal infection might be less beneficial for P transfer in plants under well watered condition than under drought condition.

Mycorrhizal symbionts are an integral part of the rhizosphere microflora, and thus contribute to the dynamic equilibrium of the rhizosphere. The increased alkaline phosphatase and dehydrogenase activities and MBC content in seedlings co-inoculated with *G. intraradices* and PSB could be due to increase in the rhizosphere microbial population as a consequence of the inoculation treatments (Aseri and Tarafdar, 2006).

From the above results, it could be concluded that co-inoculation of *G. intraradices* and PSB could be done in nursery in citrus propagation, which was found to synergistically interact to improve growth and performance of Cleopatra mandarin seedlings, much better than other microbial combinations under drought stress.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Alguacil MM, Hernández JA, Caravaca F, Portillo B, Roldán A (2003). Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil. Physiol. Plant 118:562-570.

Allen MF (2011). Linking water and nutrients through the vadose zone: a fungal interface between the soil and plant systems. J. Arid Land 3(3):155-163.

Artursson V, Finlay RD, Jansson JK (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol. 8(1):1-10.

Aseri GK, Tarafdar JC (2006). Fluorescein diacetate: a potential biological indicator for arid soils. Arid Land Res. Manage. 20:87–99.

Azcón R, Medina A, Aroca R, Ruiz-Lozano JM (2013). Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast. In: Frans JD, editor. Molecular Microbial Ecology of the Rhizosphere, Vol. 2. 1st ed. Hoboken, New Jersey, USA: John Wiley & Sons Inc Press, pp. 991-1002.

Beyer WF, Fridovich I (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Analytical Biochemistry 161: 559-566.

Bryla DR, Duniway JM (1997). Growth, phosphorus uptake and water relations of safflower and wheat infected with an arbuscular mycorrhizal fungus. New Phytologist 136 (4):581-590.

Camprubi A, Calvet C, Estaun V (1995). Growth enhancement of *Citrus reshni* after inoculation with *Glomus intraradices* and *Trichoderma aureoviride* and associated effects on microbial populations and enzyme activity in potting mixes. Plant Soil 173(2):233-238.

Caravaca F, Alguacil MM, Hernández JA, Roldán A (2005). Involvement of antioxidant enzyme and nitrate reductase activities during drought stress and recovery of mycorrhizal *Myrtus communis* and *Phillyrea angustifolia* plants. Plant Science 169:191-197.

Carlberg I, Mannervik B (1985). Glutathione reductase. Methods in Enzymology, 113:484-490.

Casida LE, Klein DA, Santoro T (1964). Soil dehydrogenase activity. Soil Science 98(6): 371-376.

Chaitanya KSK, Naithani SC (1994). Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbations during loss of viability in seeds of *Shorea robusta* Gaertn. New Phytol. 126:623-627.

Frey-Klett P, Garbaye J, Tarkka M (2007). The mycorrhizal helper bacteria revisited. New Phytol. 176:22-36.

Grifith OW (1985). Glutathione and glutathione disulphide. In: Bergmeyer HU, editor. Methods of enzymatic analysis, Vol. 8. 3rd ed. Weinheim, Germany: Verlagsgesellschaft mbH Press, pp. 521–529.

Hartree EF (1972). Determination of protein: A Modification of Lowry Method that Gives a Linear Photometric Response. Anal Biochem. 48:422-427.

Huang Z, Chao-xing H, Zhong-qun H, Zhi-rong Z, Zhi-bin Z (2010). The effects of arbuscular mycorrhizal fungi on reactive oxy-radical scavenging system of tomato under salt tolerance. Agricultural Sciences in China 9(8):1150-1159.

Kaya C, Ashraf M, Sonmez O, Aydemir S, Tuna AL, Cullu MA (2009). The influence of arbuscular mycorrhizal colonisation on key growth parameters and fruit yield of pepper plants grown at high salinity. Scientia Horticulturae 121:1-6.

Kohler J, Hernández JA, Caravaca F, Roldán A (2009). Induction of antioxidant enzymes is in volved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ Exp Bot 65: 245–252.

Luck H (1974). Catalase. In: Bergmeyer J, Grabi M, editors. Methods of Enzymatic analysis, New York, USA: Academic Press, Vol. 2:885-890.

Marulanda A, Azcon R, Ruiz-Lozano JM (2003). Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* L. plants under drought stress. Physiologia Plantarum 119:526-533.

Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22(5):867-880.

Navarro GA, Del P, Banon AS, Morte A, Sanchez-Blanco MJ (2011).

Effects of nursery pre-conditioning through mycorrhizal inoculation and drought in *Arbutus unedo* L. plants. Mycorrhiza 21:53-64.
Nunan N, Morgan MA, Herlihy M (1998). Ultraviolet absorbance (280 nm) of compounds released from soil during Chloroform fumigation as an estimate of the microbial biomass. Soil Biol Biochem 30:1599-1603.
Ouko JO, Abubaker AS (1988). Evaluation of citrus rootstocks in coast province of Kenya. Acta Hort. 218:43-50.
Phillips JM, Hayman DS (1970). Improved procedure for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions in Bazilian Mycological Society 55:158-161.
Rana A, Saharan B, Joshi M, Prasanna R, Kumar K, Nain L (2011a). Identification of multi-trait PGPR isolates and evaluating their potential as inoculants for wheat. Ann Microbiol. 61:893-900.
Rana A, Saharan B, Kabi SR, Prasanna R, Nain L (2011b). Providencia, a PGPR with biocontrol potential elicits defense enzymes in wheat. Ann Pl Prot. Sci. 19(1):138-141.
Rao MV, Paliyath G, Ormrod DP (1996). Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110:125-136.
Ruiz-Sanchez M, Armada E, Munoz Y, Salamone IEG, Aroca R, Ruiz-Lozano JM, Azcon R (2011). *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. J. Plant Physiol. 168(10):1031-1037.
Saeidnejad AH, Kafi M, Khazaei HR, Pessarakli M (2013). Effects of drought stress on quantitative and qualitative yield and antioxidative activity of *Bunium persicum*. Turkish J. Bot. 37:930-939.
Smith IK (1985). Stimulation of glutathione synthesis in photorespiring by catalase inhibitors. Plant Physiol. 79:1044-1047.
Tabatabai MA, Bremner JM (1969). Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology Biochem. 1: 301–307.
Thomas RL, Jen JJ, Morr CV (1981). Changes in soluble and bound peroxidase, IAA oxidase during tomato fruit development. J. Food Sci. 47:158-161.
Toljander JF, Artursson V, Paul LR, Jansson JK, Finlay RD (2006). Attachment of different soil bacteria to arbuscular mycorrhizal fungi is determined by hyphal vitality and fungal species. FEMS Microbiology Letters 254:34-40.
Toro M, Azcon R, Barea JM (1997). Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl. Environ. Microbiol. 63:4408-4412.
Vivas A, Azcon R, Biro B, Barea JM, Ruiz-Lozano JM (2003). Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J. Microbiol. 49:577-588.
Wu QS, Xia RX, Zou YN (2006). Reactive oxygen metabolism in mycorrhizal and non-mycorrhizal Cleopatra mandarin (*Poncirus trifoliata*) seedlings subjected to drought stress. J. Plant Physiol. 163:1101-1110.
Wu QS, Zou YN (2009). Mycorrhiza has a direct effect on reactive oxygen metabolism of drought-stressed Cleopatra mandarin. Plant Soil Environ. 55(10):436-442.