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Synergistic interaction of arbuscular mycorrhizal fungi and mycorrhiza helper bacteria improving antioxidant activities in Troyer citrange and Cleopatra mandarin under low moisture stress

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ABSTRACT

A glasshouse experiment was conducted to investigate the influence of arbuscular mycorrhizal fungi (AMF) and mycorrhiza helper bacteria (MHB) on growth, osmotic adjustment, and antioxidant activities in Troyer citrange and Cleopatra mandarin in pot culture under well-watered (WW) and water stress (WS) conditions. After 270 days of inoculation with microbial culture(s), half of the seedlings of each genotype were subjected to water stress and the other half were WW for 20 days. During the moisture stress mycorrhizal root colonization showed a durative reduction. No mycorrhizal colonization was observed in the roots of non-AM seedlings. Troyer citrange and Cleopatra mandarin recorded significantly highest root colonization just before imposing differential water treatment (83.34 and 80.00%, respectively) and after 20 days of differential water treatment (66.67 and 62.50%, respectively) under WS and (87.50 and 83.33%, respectively) under WW, when dually inoculated with *Glomus intraradices* and PSB. *Glomus intraradices* in association with PSB or *Azospirillum* accumulated higher concentration of osmolytes like total phenols, proline and total soluble sugars and antioxidant metabolites (carotenoids and ascorbic acid) in leaves of citrus seedlings under WS.

Key words: Citrus rootstocks, *Glomus intraradices*, moisture stress, osmo-regulation, antioxidant metabolites.

INTRODUCTION

Water deficit is an important biotic factor limiting plant growth and yield in many areas on the earth that is increasingly topical because of climate change and water shortages (Jafarzadeh and Abbasi, 10) and thus cause the economic losses in agriculture. Citrus is one of the most important commercial fruit crops grown all over the world, however, moisture deficit condition restrict the yield and quality of citrus fruits in dryland ecosystems. Citrus has few and short root hairs in the field and is highly dependent on arbuscular mycorrhizae (AM), because the most common mutualistic symbiosis replaces part of the root-hair functions (Graham and Syvertsen, 7). It is well known that a considerable number of plant growth promoting bacteria producing phytohormones cohabit in the rhizosphere with AM fungi and could play a helper role in the plant-fungus interaction (Medina *et al*., 14). Thus, there is a growing interest to evaluate the abilities of specific fungus-bacterium-plant associations to alleviate drought tolerance to enhance crop production in arid ecosystem. Therefore, the present study was undertaken on two citrus rootstocks (Troyer citrange and Cleopatra mandarin) to determine the effectiveness of AM fungi and mycorrhizal helper bacteria in osmotic adjustment and tolerance of plants against oxidative damage under moisture stress conditions.

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MATERIALS AND METHODS

The pot experiment was conducted on moderately moisture stress tolerant citrus rootstocks Troyer citrange (*Citrus sinensis* × *Poncirus trifoliata*) and Cleopatra mandarin (*C. reshni*) to understand the physiological response of plants to different microbial inoculants under differential soil moisture status. Seeds of these rootstocks collected from the Citrus Germplasm Block of the Division of Fruits and Horticultural Technology, IARI, New Delhi, were surface sterilized by immersing in 70% alcohol for five minutes, followed by rinsings with sterile distilled water and then germinated on wet filter paper in Petri dishes at 28°C. After 7 days these seedlings were transplanted in plastic containers (12 cm × 20 cm) containing mixture of soil: sand: FYM (2:2:1) under glasshouse conditions. The AMF strain *Glomus intraradices* and three mycorrhizal helper bacterial strains, *viz*., phosphate solubilising bacteria (*Bacilus subtilis* + *B. megatherium*), *Azospirillum* and *Providencia* were used in the experiment. The mycorrhizal treatment consisted of 20 g inocula per treatment per pot. The arbuscular mycorrhiza (AM) fungi and helper bacteria alone or in combination were applied at 5 cm below the seedlings so that AM spores upon germination can infect the fine citrus roots after proper root formation and thus penetrate into host's parenchyma cortex. The seedlings were maintained in a glasshouse with day and night temperature ranging from 27 ± 1°C. Day lengths were extended upto 16 h with cool white fluorescent lights at 630 μmol $m^{-2}s^{-1}$. Humidity was maintained at 80-85 per cent using an electronic automatically controlled humidifier. Seedlings were watered on alternate day.

The differential water treatment was started at 270 days after inoculation (DAI) under glasshouse conditions. The citrus seedlings in each replication of each treatment (six seedlings per replication per treatment) were divided into two equal sets and each set was subjected to either water stress or well watered condition, during which soil relative water content was measured using soil moisture meter fitted with 4.7 inches probes. Wet point was fixed at 90% and dry point at 8%. The soil relative water content (RWC) for WW plants was monitored at 80%. Duration of withholding irrigation was 20 days, when more than 50% treatment under water stress condition, showed the visible symptoms of temporary wilting. Entire plants were harvested after 20 days and some samples, immediately after washing with double-distilled water were stored at -20°C deep freeze for estimation of antioxidant enzymes and reactive oxygen species.

Observations were recorded after 20 days of differential water treatment for water stressed (WS) plants and well-watered (WW) plants. The percentage of AM colonization in roots was analyzed (Philips and Haymans, 18). Leaf proline content was estimated as per the method of Bates *et al*. (3). Total phenols content in leaf was estimated according to Malik and Singh (12). Total soluble sugars of leaf was estimated and compared with the prepared standard curve by the method as suggested by Buysse and Merckx (4). The antioxidant metabolites like ascorbic acid was estimated as described by Mukherjee and Choudhuri (15). The absorbance was recorded at 530 nm. Total carotenoids was determined as per the method suggested by by Hiscox and Israelstam (9).

The experimental design adopted was completely randomised design with 16 treatments and two replications per treatment. The treatment included T_1 = Troyer + control, T_2 = Troyer + phosphate solubilising bacteria (PSB), T_3 = Troyer + *Azospirillum* (*Azo*), T_4 = Troyer + *Providencia*, T_5 = Troyer + *Glomus intraradices* (*G. i.*) T_6 = Troyer + *G. i.* + PSB, T_7 = Troyer + *G. i.* + *Azo*, T_8 = Troyer + *G. i.* + *Providencia*, T_9 = Cleopatra mandarin + control, T_{10} = Cleopatra mandarin + PSB, T_{11} = Cleopatra mandarin + *Azo*, T_{12} = Cleopatra mandarin + *Providencia*, T_{13} = Cleopatra mandarin + *G. i.*, T_{14} = Cleopatra mandarin + *G. i.* + PSB, T_{15} = Cleopatra mandarin + *G. i.* + *Azo*, T_{16} = Cleopatra mandarin + *G. i.* + *Providencia*. The two year experimental data were analyzed with SPSS package (SPSS 11.0) and significance of variance was estimated by applying F test at 5% level of significance.

RESULTS AND DISCUSSION

The water stressed (WS) treatment notably decreased root colonization. No mycorrhizal colonization was observed in the roots of non-AM seedlings. However, dual inoculated treatment had significantly highest root colonization, as compared to single inoculation, particularly *G. intraradices* + PSB, under WS. Troyer citrange and Cleopatra mandarin recorded significantly highest root colonization for both WS (66.67 and 62.50%, respectively) and WW plants (87.50 and 83.33%, respectively), when dually inoculated with *Glomus intraradices* and PSB (Table 1; Fig. 1). These results are in consonance with Artursson *et al*. (2) who also found that mycorrhizal helper bacteria can stimulate the pre-symbiotic fungal growth, which leads to an increase in root-fungus contacts and root colonization.

When plant is subjected to drought stress, leaf water potential decreases so as to maintain

Table 1. Effect of microbial inoculants on root colonization of Troyer citrange and Cleopatra mandarin at differential soil moisture regimes.

Treatment	Differential moisture regime	
	Water stressed (WS)	Well-watered WW)
T_1	0.00 (0.83)	8.33 (16.77)
T_2	0.00 (0.83)	16.66 (23.39)
T_3	0.00 (0.83)	16.67 (24.10)
T_4	0.00 (0.83)	12.50 (20.44)
T_5	54.17 (47.47)	75.00 (60.00)
T_6	66.67 (54.74)	87.50 (69.56)
T_7	62.50 (52.27)	83.33 (65.90)
T_8	58.33 (49.79)	79.16 (62.95)
T_9	0.00 (0.83)	8.33 (16.77)
T_{10}	0.00 (0.83)	20.84 (27.05)
T_{11}	0.00 (0.83)	16.66 (23.39)
T_{12}	0.00 (0.83)	16.67 (24.10)
T_{13}	33.33 (35.26)	70.84 (57.37)
T_{14}	62.50 (52.27)	83.33 (65.90)
T_{15}	37.50 (37.73)	79.16 (62.95)
T_{16}	41.67 (40.21)	75.00 (60.00)
$CD_{0.05}$	9.99	10.89

*Figures in parenthesis are Arc Sin √% transformation data.

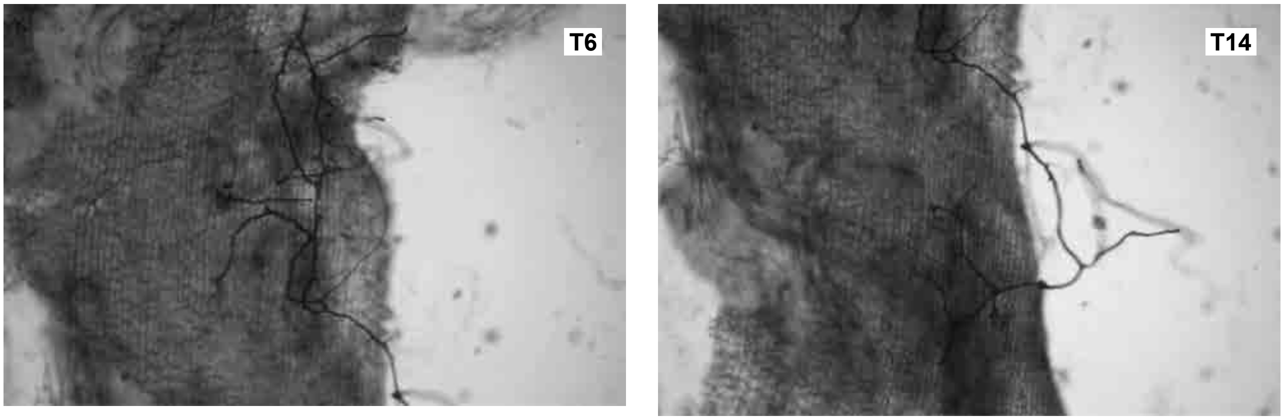

Fig. 1. Root colonization of Troyer citrange and Cleopatra mandarin inoculated with *Glomus intraradices* and PSB under WS conditions.

a favourable gradient for water flow from soil into roots. Osmoregulation, defined as lowering of the osmotic potential due to net solute accumulation in response to water stress, may help to preserve the metabolic processes and is regarded as a beneficial drought-tolerance mechanism (Martinez *et al*., 13). The present investigation revealed that co-inoculation of *G*. *intraradices* and *Azospirillum* significantly accumulated higher amount of total phenols in leaf of Troyer citrange (46.87 and 36.70 mg 100 g^{-1}) and Cleopatra mandarin (46.14 and 34.91 mg 100 g^{-1}) under WS and WW conditions, respectively (Table 2). Similar result was obtained by Eftekhari*et al*. (5) who found that grapevines when inoculated with beneficial micro-organisms had significantly higher content of total phenols in the foliage as compared to un-inoculated control, indicating enhancement in defense mechanism of inoculated plants against diseases. Proline is used to be an indicator of drought tolerance and plays a role in the osmotic adjustment phenomenon. In the present investigation, dual inoculation of *G*. *intraradices* and PSB caused significantly increased level of leaf proline in Troyer citrange (178.30 and 90.78 µg g^{-1}) and in Cleopatra mandarin (184.18 and 97.94 µg g^{-1}) under WS and WW conditions, respectively (Table 2). The increasing proline accumulation in AM fungi and helper bacteria inoculated citrus seedlings under water stress could induce the adjustment of cell osmotic potential, indicative of osmotic cellular adaptation. This is a mechanism by which microbial cells can cope with drought stress (Paleg *et al*., 17). Similar result was found by Kandowangko *et al*. (11). Troyer citrange seedlings inoculated with *G*. *intraradices* + PSB significantly recorded highest total soluble sugars in leaf (44.59 and 34.57 per cent) under WS and WW conditions, respectively. In case of Cleopatra mandarin, the highest total soluble sugars was found in *G*. *intraradices* + PSB (47.49 and 36.63 per cent) under WS and WW conditions, respectively (Table 2). The results indicated that photosynthetic carbohydrate, especially total soluble sugars, were conducive to be retained in seedlings by AM colonization. The result is

Table 2. Effect of microbial inoculants on osmolyte content of Troyer citrange and Cleopatra mandarin seedlings at differential soil moisture regimes.

Treatment	Total phenols (mg 100 g^{-1})		Proline (µg g^{-1})		Total soluble sugars (%)	
	WS	WW	WS	WW	WS	WW
T_1	21.09	14.95	53.70	40.64	26.63	13.05
T_2	22.61	17.37	61.44	54.42	26.89	14.34
T_3	22.12	18.07	68.32	54.94	33.52	15.57
T_4	22.56	16.89	81.70	65.72	31.54	14.70
T_5	34.81	26.97	91.10	73.50	33.45	27.42
T_6	43.21	33.67	178.30	90.78	44.59	34.57
T_7	46.87	36.70	122.30	77.82	38.87	30.38
T_8	41.21	30.80	131.10	76.16	37.49	29.53
T_9	19.48	12.30	58.80	43.26	28.67	17.45
T_{10}	21.10	13.56	66.60	57.34	28.94	18.06
T_{11}	21.58	14.80	73.50	56.20	35.39	19.61
T_{12}	21.09	15.56	83.76	69.78	37.39	20.39
T_{13}	33.98	24.82	94.80	75.56	39.31	31.44
T_{14}	42.13	31.20	184.18	97.94	47.49	36.63
T_{15}	46.14	34.91	124.18	80.54	42.63	32.45
T_{16}	39.03	29.40	131.68	79.80	40.21	30.54
$CD_{0.05}$	0.91	0.87	0.89	1.23	0.23	0.35

WS = Water stress, WW = Well-watered.

in harmony with the findings of Abbaspour *et al*. (1) in *Pistacia vera*, which may be due to the sink effect of the mycorrhizal fungus demanding sugars from shoot tissues (Wright *et al*., 20).

Water stress, among other changes, has the ability to reduce the tissue concentrations of antioxidant like carotenoids, primarily with the production of reactive oxygen species in the thylakoids. In response to oxidative stress, plants have the defensive mechanism to scavenge the reactive oxygen species. In the present investigation, decrease in total carotenoids content in citrus leaves was noticed as a result of water stress. One reason for decrease in carotenoids during osmotic stress might be Beta-carotene destruction and zea-xanthin formation (Sultana *et al*., 19). The present investigation revealed that *G*. *intraradices* + PSB caused significant accumulation of total carotenoids in leaf (0.80 and 0.65 mg g^{-1}) in Troyer citrange and Cleopatra mandarin, respectively, under WS condition, as compared to other treatments, including un-inoculated control (Fig. 2). The result of this study is consistent with the results obtained from *Ocimum basilicum* (Enteshari and Hajbagheri, 8). The higher content of total carotenoids in *G*. *intraradices* + PSB-inoculated citrus seedlings has been attributed to the increase in AMF symbiosis by the specific helper bacteria, thereby increasing fungal hyphal mats and extension of hypha with a diameter of 2-5 µm to penetrate soil pore inaccessible to root hairs (10-20 µm) and so absorb higher quantity of soil moisture (Gong and Zhong, 6), resulting in lesser production of reactive oxygen species in the leaves, as carotenoids destruction is related to oxygen produced in thylakoid (Munne-Bosch and Penuelas, 16). The present study revealed that *G*. *intraradices* + *Providencia* had significant influence on increase in ascorbic acid content in leaves of both Troyer citrange and Cleopatra mandarin seedlings (250.50 and 237.50 µmol g^{-1}, respectively) under WS condition. Under WW condition, *G*. *intraradices* and PSB treated seedlings exhibited significant increase in ascorbic acid concentration in leaves of Troyer citrange and Cleopatra mandarin (363.50 and 342.50 µmol g^{-1}, respectively) (Fig. 2). Ascorbic acid, an anti-oxidant, is responsible for non-enzymatic scavenging of super oxide radical and regeneration of α-tocopherol in the chloroplast. The present result confirms the findings of Wu and Zou (2009) who found that the contents of non-enzymatic antioxidants of AM citrus trees were positively correlated with AM colonization.

On the basis of findings of the present investigation, it can be concluded that citrus seedlings dually inoculated with AM fungi *G. intraradices* and mycorrhizal helper bacteria (PSB, *Azospirillum* or *Providencia*) maintained higher content of osmolytes (total soluble sugars, phenols and proline) and antioxidant metabolites (ascorbic acid and carotenoids). In particular, *G*. *intraradices* and PSB (*Bacillus subtilis* + *B*. *megatherium*) treatment exhibited significant superiority, over other microbial treatments, in terms of physical, physiological and biochemical status of citrus seedlings. Thus, dual inoculation of *Glomus intraradices* and PSB could be done in nursery in plant propagation and improving seedling vigour and health, rather use of expensive fertilizers.

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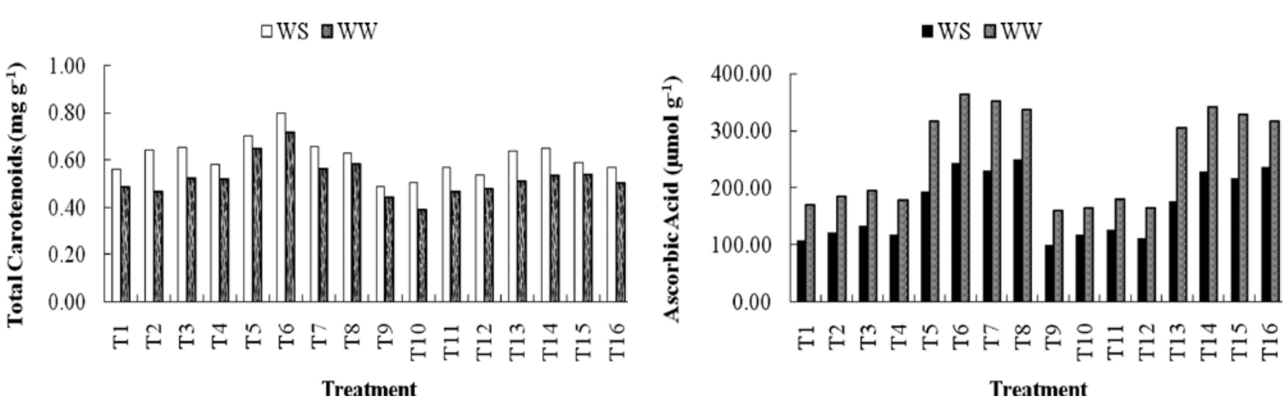

Fig. 2. Effect of microbial inoculants on antioxidant properties (total carotenoids & ascorbic acid) in leaf of Troyer citrange and Cleopatra mandarin at differential soil moisture regimes.

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