Physiological response of *Vicia faba* L. to inoculation with *Rhizobium* and arbuscular mycorrhizal fungi: Comparative study for irrigation with Nile water and wastewater

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Abstract

Field experiments were carried out to investigate the effects of *Rhizobium* (R), arbuscular mycorrhizal fungi (AMF) inoculation individually and in combination, on chlorophyll, soluble proteins, sugars, and indole acetic acid (IAA) contents, and polyphenol oxidase (PPO) activity of faba bean plant. The analyses of physiological parameters have been recorded as influenced by irrigation with Nile water (clear water) and wastewater (heavy metals polluted water). Heavy metals (HMs) comprising Mn and Cr were found to be higher than acceptable limits in wastewater used to irrigate crops around Bahr El-Baqar drainage canal (Abu-Hammad, Sharkia, Egypt). The highest content of chlorophyll, protein, soluble and insoluble sugars was observed with a combined R+AMF inoculant throughout the different stages of faba bean growth irrigated either with Nile water or wastewater. Regarding to IAA, the AMF inoculation (either alone or in combination with R) was generally more efficient in IAA induction than the R inoculation. Meanwhile, the latter inoculation was superior only in plants irrigated with Nile water at the vegetative stage and plants irrigated with wastewater at the fruiting stage. Reduced levels of both chlorophyll and soluble protein were recorded in plants irrigated with wastewater compared to those irrigated with Nile water. However, the activity of PPO was found to be higher in the plants irrigated with wastewater than those irrigated with Nile water. Results of mycorrhizal colonization and number of formed root nodules indicated that AMF and R have a notable tolerance to heavy metals polluted water. This study suggests that the co-inoculation with R+AMF could be used in alleviation of HMs toxicity to plants and revegetation on HMs-contaminated soils or wastewater-irrigated soils.

Keywords: Faba bean, Arbuscular mycorrhizal fungi, *Rhizobium*, Physiological analyses, Nile water, Wastewater, Heavy metals toxicity.
Abbreviations: AMF_Arbuscular mycorrhizal fungi; R_*Rhizobium*; HMs_Heavy metals; Chl a_Chlorophyll a; Chl b_Chlorophyll b; Indole acetic acid_IAA; Polyphenol oxidase_PPO; ROS_Reactive oxygen species.

Introduction

Water pollution is a very important environmental problem and has been drawing considerable public attention over the last few decades. The quantity and diversity of toxic and hazardous wastes have been increased with the increase in quick industrial development and urbanization (Abdullah, 1995). The unscientific disposal of untreated or undertreated sewage, agricultural and industrial effluents results in the accumulation of heavy metals (HMs) in land and water bodies (Purakayastha and Chhonkar, 2010). HMs have a significant toxic effect on human, animals, microorganisms and plants (Majid et al., 2012). Excessive accumulation of HMs in agricultural soils, through wastewater irrigation, may not only result in soil contamination, but also affects the food quality and safety (Muchuweti et al., 2006). HMs taken up by plants from contaminated soil and water operate as stress factors causing physiological constraints where they reduce growth and impair its metabolism (Lata, 1989). The plant-metals response results in the production of reactive oxygen species (ROS). Consequently, oxidative stress and lipid peroxidation occur. To cope with ROS and alleviate their toxic effects, plant cells are equipped with a well-developed antioxidative system (Yamamoto et al., 2003). Polyphenol oxidase (PPO) is one of the most important antioxidants, its activity has been shown to increase under HMs stress and has thus been associated to some form of defence mechanism

(Martins and Mourato, 2006). Since, it has been well documented that the conventional methods used for metal detoxification are laborious, expensive and severely alter the agroecosystem balance (Perelo, 2010; Sharma, 2012); there is a growing worldwide demand for ecologically compatible environmentally friendly techniques in agriculture, capable of providing adequate nourishment for the increasing human population and improving the quality and quantity of certain agricultural products. Hence, the application of beneficial microorganisms is an important alternative technique. For instance, plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) have been used in several trials (Horswell et al., 2006; Teng et al., 2010). They have been reported to play significant roles in recycling of plant nutrients, maintenance of soil structure, and detoxification of noxious chemicals and control of plant pests (Filip, 2002). PGPR include a diverse group of free-living soil bacteria that can improve host plant growth and development in HMs-contaminated soils by mitigating toxic effects of HMs on plants (Zhuang et al., 2007). A list of PGPR associated with plants grown in metal-contaminated soils like *Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *Bacillus mucilaginosus* HKK-1, *Bacillus subtilis* SJ-101, *Brevundimonas* spp. KR013, *Pseudomonas fluorescens* CR3, *Rhizobium leguminosarum* bv. *trifolii*

NZP561 are used in bioremediation (Zhuang et al., 2007). Mycorrhizae have been reported in plants growing on HM-contaminated sites (Shetty et al., 1995) indicating that these fungi have evolved a HMs tolerance and that they may play a role in the phytoremediation of the site. Different researchers have found that most of the plants growing in HM-polluted sites were associated with *Glomus* and *Gigaspora* mycorrhizal fungal taxa (Weissenhorn and Leyval, 1995; Khan et al., 2000). AMF and *Rhizobium* (R) have been reported to be key elements for plant establishment under xeric and nutrient-unbalanced conditions (Requena et al., 1996). Optimum growth of leguminous plants is usually dependent on such symbiotic relationships with AMF and N_2-fixing bacteria (Xavier and Germida, 2003). Faba bean (*Vicia faba* L.) is one of the most important winter crops of high nutritive value in the world as well as in Egypt. Mature seeds of faba bean are good sources of protein (about 25% in dried seeds), starch, cellulose, vitamin C and minerals. High yield, smaller seeds, less anti-nutritional factors, high adaptation ability to modern agriculture, the longevity of storage life, ease of transportation and their low cost make this plant more attractive for farmers, feed and food manufactures (Duc, 1997). Due to these benefits including the high nutritional value and energy producing property of this plant and the urgent need to improve its yield and vegetation, the present study was undertaken to investigate the impact of inoculation by R bacteria and AM fungi on some physiological parameters of the faba bean plants irrigated with wastewater (HMs-polluted water) and Nile water (clear water).

Results and discussion

Response of physiological and biochemical parameters of Vicia faba to R and AMF inoculants as influenced by irrigation with Nile water and wastewater

Response of leaf chlorophyll content

Tables (2) and (3) showed that throughout the different growth stages of faba bean plants irrigated either with Nile water or wastewater, the dual inoculation with R+AMF has significantly enhanced the photosynthetic capacity by increasing the Chl. a and Chl. b contents of the plant over other treatments including uninoculated control treatment. The highest total chlorophyll content was recorded at the flowering stage. At this stage, with irrigation by Nile water, the plants inoculated dually with R+AMF showed an increase in chlorophyll content by 13.1%, 12.1%, and 9.70% higher than that recorded in inoculated plants of control (Nile water without treatment), R, and AMF, respectively. However, with irrigation by wastewater, the plants inoculated dually with R+AMF showed an increase in chlorophyll content by 16.6%, 13.9%, and 9.70% higher than that recorded in plants inoculated singly with the respective treatments. The dual inoculation was reported to increase the total chlorophyll content of leaves of tested plants compared to those of control (without inoculation) and single inoculation with R and AMF (Bhattacharjee and Sharma, 2012). This increase may be due to an increase in stomatal conductance, photosynthesis, transpiration and enhanced plant growth (Rajasekaran et al., 2006) or due to the presence of large and more numerous bundle sheath chloroplasts in the inoculated leaves (Krishna and Bagyaraj, 1984). Also, the single

inoculation either with R or AMF with Nile water and wastewater irrigation (Tables 2 and 3) showed stimulatory effect on the Chl. a and Chl. b contents but with little efficiency than the co-inoculation. Similar findings were obtained by Bhattacharjee and Sharma (2012) studies on pigeon pea plant. The increase in chlorophyll content in inoculated plants with R and AMF has been reported to meet the carbon requirements from their host plants (Sivaprasad and Rai, 1987; Lalitha and Santhaguru, 2012). The highest pigment content was recorded at flowering stage compared to both vegetative and fruiting stages. This was interpreted on the basis of enhanced vegetative growth and the better nutrient acquisition by symbioses during the flowering stage (Kaschuk et al., 2010). It was obvious from Tables (2) and (3) that irrigation with wastewater resulted in reduced Chl. a and Chl. b contents in the faba bean plants compared to those irrigated with Nile water. Findings were supported by those reported by Shanker et al. (2005) who reported the disorganization of the chloroplast ultrastructure and inhibition of electron transport processes in the plants grown under high concentration of HMs.

Response of soluble protein content

Table (4) showed that with irrigation of faba bean plants either with Nile water or wastewater, the co-inoculation with R+AMF has significantly increased the protein content over different treatments and control at all growth stages. At the flowering stage, with Nile water irrigation, the dual inoculation of R+AMF showed an increase of the soluble protein concentration by 49 and 7.40 %, higher than those of dual inoculation at vegetative and fruiting stages, respectively. However, with wastewater irrigation, the soluble protein content resulted from dual inoculation of R+AMF exceeded those expressed at vegetative and fruiting stages by 97.1 and 28.1 %, respectively. Morad et al. (2013) reported that the plants with higher total chlorophyll content showed a higher leaf protein content which is a good index for better biological nitrogen absorption. These results are similar to those obtained by Afzal and Bano (2008) who reported that the leaf protein was significantly increased by rhizobial treatments. Moreover, Lenin et al. (2010) reported that the protein content increased in AMF inoculated when compared to non-mycorrhizal seedlings (control). The soluble protein content of the plants irrigated with wastewater was lower than those irrigated with Nile water at different harvest stages, as shown in Table (4). Decrease in the protein content has also been found in *Brassica juncea* grown on various amendments of tannery waste containing HMs (Singh and Sinha, 2005). The decrease in protein content in faba bean plants irrigated with wastewater may be interpreted on the basis of enhanced protein degradation process as a result of increased protease activity. This was previously observed by Palma et al. (2002) at higher concentrations of Cd and Pb in *B. juncea*. The HMs may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of ROS which led to reduced protein content (John et al., 2009).

Response of carbohydrate content

The soluble and insoluble carbohydrate fractions were measured in the dry faba bean leaves. The results represented in Fig. 1 (A, B, C and D) indicated that wastewater irrigation reduced the soluble sugars contents compared to those obtained

Table 1. HMs concentrations in Nile water and wastewater and the guidelines for the safe limits of HMs in the irrigation water.

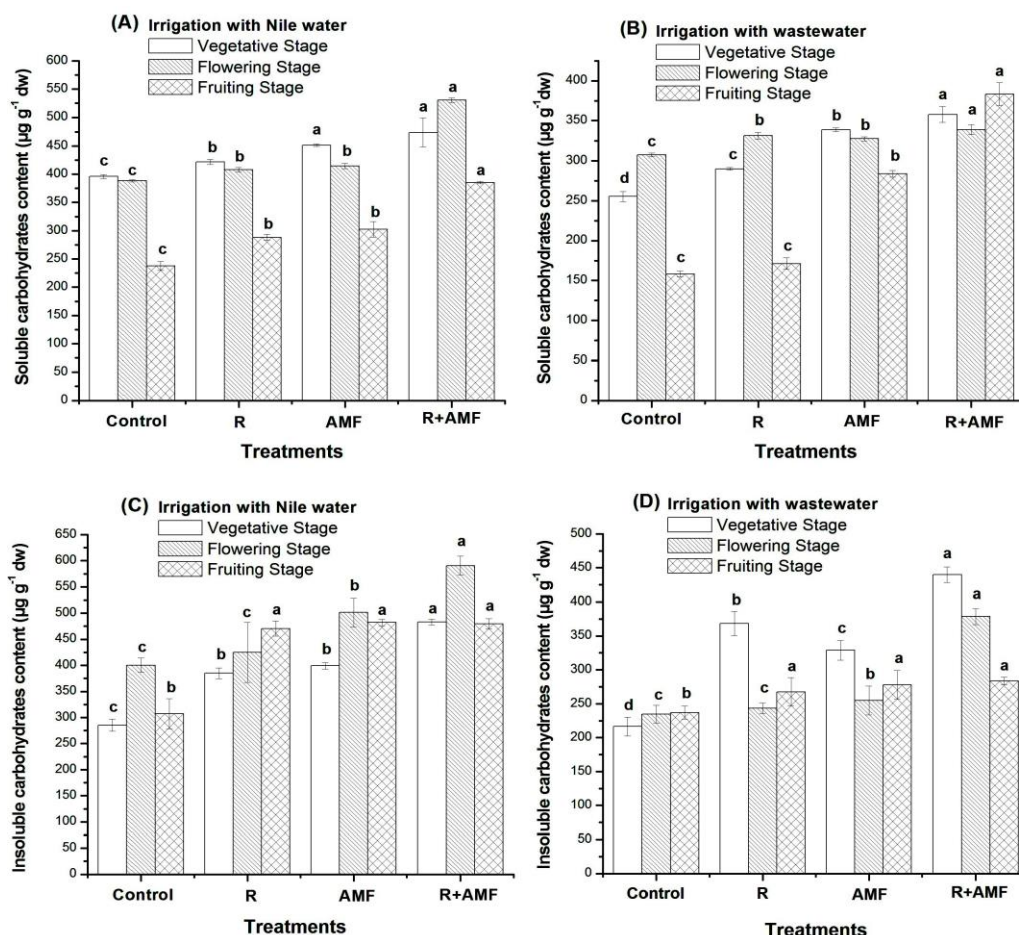| Element	Concentration in Nile water (ppm)	Concentration in wastewater (ppm)	Guideline for safe limits (ppm) (FAO, 1985)
Mn	0.07	2.98	0.20
Cr	0.006	1.49	0.10
Ni	0.002	0.07	0.20
Zn	0.081	0.14	2.00
Pb	0.063	0.07	5.00
Cu	0.010	0.11	0.20
Co	0.001	0.27	5.00
Cd	N.D.	0.003	0.01

N.D., not detected

Fig 1. Effect of R and AMF inoculants on soluble and insoluble carbohydrate content ($\mu g\ g^{-1}$ dw) of faba bean leaves at different growth stages. Soluble carbohydrate fraction of leaves irrigated with Nile water (A), soluble carbohydrate fraction of leaves irrigated with wastewater (B), insoluble carbohydrate fraction of leaves irrigated with Nile water (C), and insoluble carbohydrate fraction of leaves irrigated with wastewater (D). Data are shown as the mean ± SD of triplicate measurements from two independent seasons. Different letters for each growth stage at different treatments are statistically different (LSD test, $P \leq 0.05$).

from Nile water irrigation. This result corroborates with the results of Tzvetkova and Kolarov (1996). They reported that concentrations of total soluble sugars and starch decreased significantly in the trees from the polluted regions and attributed this to the photosynthesis inhibition or stimulation of respiration rate. Fig. 1 (A, B, C and D) revealed that throughout the plant growth and regardless the type of water used for irrigation, the dual inoculation with R+AMF has predominantly increased the soluble and insoluble carbohydrate fractions significantly over the singly inoculated and uninoculated control plants. The dual inoculation was reported to significantly increase the total carbohydrates over the uninoculated plants (Ramakrishnan

and Lenin, 2010). Compared to the uninoculated control plants, the single inoculation either with R or AMF also has increased the soluble and insoluble carbohydrate contents. The AMF inoculation has increased the soluble carbohydrate fraction over the R inoculated and control plants except at the flowering stage of plants irrigated with wastewater, the R inoculation caused non-significant increase over the AMF. At the vegetative stage, the R inoculated plants irrigated with wastewater showed significant increase in the insoluble carbohydrate fraction. AMF showed enhancement of the soluble and insoluble sugars of *Avena sativa* (Kumar et al., 2012), *Euterpe edulis* and *Archontophoenix alexandrae* (Sgrott et al., 2012). This enhancement was reported to be

Table 2. Effect of R and AMF inoculants on Chl. a content of faba bean leaves irrigated with Nile water and wastewater at different growth stages. Means ± SD of triplicate measurements from two independent seasons are shown.

Chl. a fraction (µg g^{-1} fw)						
Irrigation water used	Nile water			Wastewater		
Growth stage / Treatment	Vegetative	Flowering	Fruiting	Vegetative	Flowering	Fruiting
Control	1495.3 ± 3.51[d]	1832.7 ± 3.78[b]	1127.0 ± 3.60[d]	1263.7 ± 4.04[d]	1535.0 ± 6.08[d]	999.0 ± 4.58[d]
R	1600.0 ± 36.0[b]	1852.0 ± 3.60[b]	1408.7 ± 4.93[c]	1282.0 ± 4.35[c]	1580.3 ± 2.50[c]	1227.0 ± 1.73[c]
AMF	1583.7 ± 5.50[c]	1886.7 ± 2.51[b]	1653.3 ± 1.52[b]	1319.3 ± 1.15[b]	1635.7 ± 0.57[b]	1472.3 ± 4.04[b]
R+AMF	1680.0 ± 10.0[a]	2093.3 ± 83.2[a]	1930.0 ± 5.56[a]	1530.3 ± 1.52[a]	1835.7 ± 3.78[a]	1647.0 ± 2.64[a]

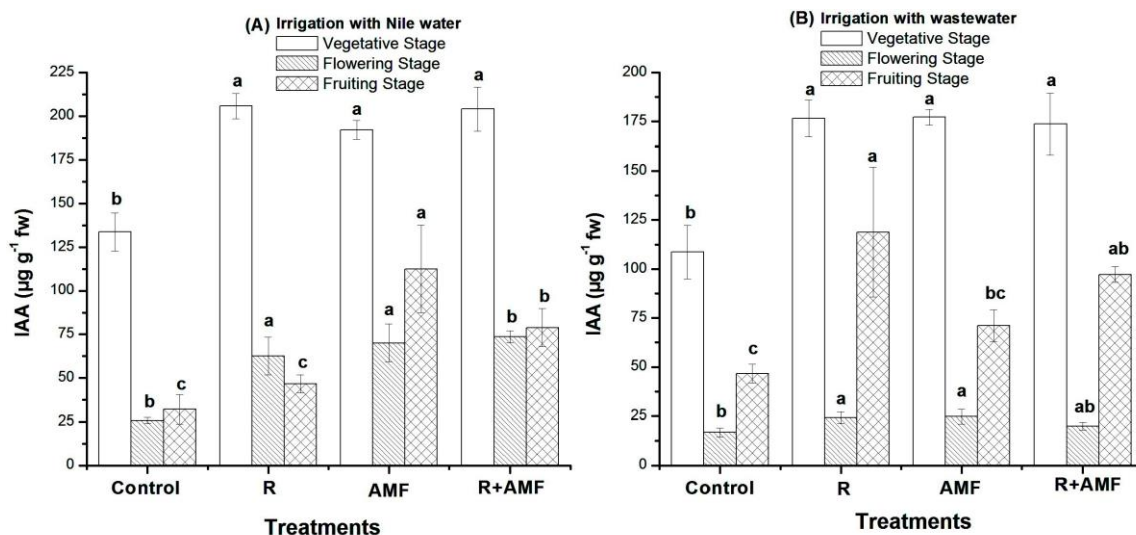[a-d]means with different superscripts in the same column are considered statistically different (LSD test, P ≤ 0.05).

Fig 2. Effect of R and AMF inoculants on IAA (µg g^{-1} fw) of faba bean shoots irrigated with Nile water (A) and wastewater (B) at different growth stages. Data are shown as the mean ± SD of triplicate measurements from two independent seasons. Different letters for each growth stage at different treatments are statistically different (LSD test, P ≤ 0.05).

correlated with the increase in photosynthetic pigments to meet the carbon requirements of AMF from their host plants (Sivaprasad and Rai 1987; Lalitha and Santhaguru, 2012). Also, R inoculation was reported to significantly increase leaf sugar content (Afzal and Bano, 2008).

Response of IAA content

IAA is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation, pigment formation, biosynthesis of various metabolites and responses to light and gravity (Taiz and Zeiger, 1998). Fig. 2 (A and B) revealed that inoculation with R and AMF either singly or dually had a stimulatory effect on the IAA production over the control uninoculated plants. However, the mycorrhizal inoculation (either alone or in combination with R) was more efficient in IAA induction than the R inoculation which was superior only in plants irrigated with Nile water at the vegetative stage that recorded the highest IAA content among the different treatments (205.71 µg IAA g^{-1} fresh weight) and plants irrigated with wastewater at the fruiting stage (118.86 µg IAA g^{-1} fresh weight). Similarly, Scagel (2003) attributed the altered plant (Freesia) development in response to AMF inoculation to some sort of signaling occurs between the fungus or microorganisms in the inoculums and the plant. He further reported that mycorrhizal fungi induce production of cytokinins, IAA and ethylene so it is possible that AMF may

alter aspects of Freesia development including shoot emergence and flowering through hormonal signals between the fungus and the plant. Torelli et al. (2000) found in mycorrhizal onion plants an IAA increase, similarly to that observed in faba bean plants used in our study. Also, the increase of IAA concentration observed in *"Glomus". fasciculatum* inoculated *Prosopis juliflora*, recorded by Selvaraj (1998), affirmed the influence of AMF on increased level of growth hormones. R bacteria were reported to synthesize phytohormones like auxin as secondary metabolites in inoculated plants (Seneviratne et al., 2000).

Response of PPO activity

Data obtained in Table (5) indicated that the co-inoculation of faba bean plants with R+AMF has significantly enhanced the activity of PPO. At the vegetative stage, the PPO activity was 16 and 24 U g^{-1} fresh weight in the plants irrigated with Nile water and wastewater, respectively. However, in flowering stage, the activity was 20 and 40 U g^{-1} fresh weight in plants irrigated with the respective water types. The present results concur with those of Devi and Reddy (2002). They observed increases in the activity of peroxidase and PPO following inoculation of groundnut with R and AMF. The present study further showed that AMF inoculated plants had a superior PPO activity over the R inoculated and uninoculated (control) plants. In accordance with these results, Aziz et al. (2011) reported that PPO activity has

Table 3. Effect of R and AMF inoculants on Chl. b content of faba bean leaves irrigated with Nile water and wastewater at different growth stages. Means ± SD of triplicate measurements from two independent seasons are shown.

Chl. b fraction ($\mu g\ g^{-1}$ fw)

Irrigation water used	Nile water			Wastewater		
Growth stage Treatment	Vegetative	Flowering	Fruiting	Vegetative	Flowering	Fruiting
Control	618.67 ± 2.08[c]	862.67 ± 2.88[c]	626.33 ± 2.30[d]	525.33 ± 3.05[b]	629.67 ± 4.50[c]	518.33 ± 4.61[d]
R	623.00 ± 4.00[bc]	868.00 ± 4.58[c]	732.33 ± 4.04[b]	533.33 ± 7.57[b]	633.00 ± 2.64[c]	570.00 ± 1.00[c]
AMF	629.00 ± 6.08[b]	892.67 ± 3.05[b]	710.00 ± 4.00[c]	573.33 ± 1.52[a]	664.67 ± 4.72[b]	600.00 ± 6.24[b]
R+AMF	672.00 ± 3.46[a]	958.67 ± 8.08[a]	815.67 ± 3.51[a]	577.67 ± 2.08[a]	687.33 ± 4.04[a]	622.67 ± 6.80[a]

[a-d] means with different superscripts in the same column are considered statistically different (LSD test, P ≤ 0.05).
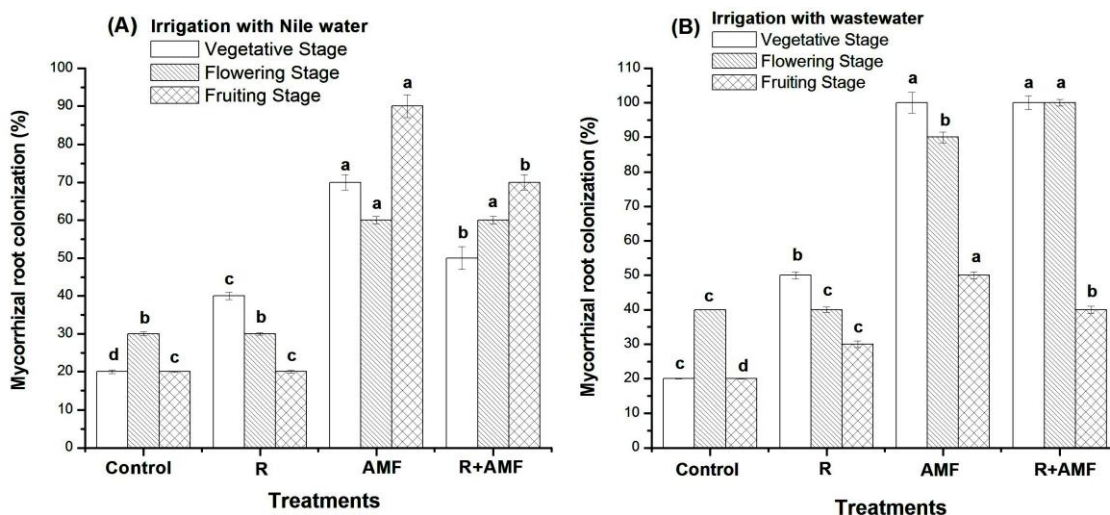

Fig 3. Effect of R and AMF inoculants on mycorrhizal colonization (%) of faba bean roots irrigated with Nile water (A) and wastewater (B) at different growth stages. Calculated mean is for 10 samples from two independent experiments ± SD. Different letters for each growth stage at different treatments are statistically different (LSD test, P ≤ 0.05).

increased significantly in mycorrhizal *Solanum* plants than non-mycorrhizal ones. The PPO activity was found to be higher in the plants irrigated with wastewater than those irrigated with Nile water. Similarly, Polovnikova and Voskresenskaya (2008) reported that PPO activity was enhanced with increased anthropogenic medium pollution and this might be due to the enhanced PPO activity that serves as an additional plant defense, detoxifying anthropogenic pollutants. This allows the maintenance of oxidative metabolism at a definite stable level and results in improved plant adaptive capacities (Mittler, 2002; Martins and Mourato, 2006).

Response of mycorrhizal colonization and rhizobial nodulation of Vicia faba roots to R and AMF inoculants as influenced by irrigation with Nile water and wastewater

Fig. 3 (A and B) showed that the mycorrhizal colonization of faba bean plants irrigated either with Nile water or wastewater, varied among the harvest stages. In general terms, it was observed that single inoculation with AMF had the highest mycorrhizal colonization either in plants irrigated with Nile water or wastewater along the different harvest stages except at vegetative and flowering stages in plants irrigated with wastewater, the dual inoculation with R+AMF was the superior recording 100% mycorrhizal colonization. This value was then declined to 40% by the last harvest (Fruiting stage). Similarly, Zhang et al. (1995) found that the mycorrhizal colonization increased in soybean roots at the first harvest until the flowering stage, after which it decreased

and attributed that increase to nitrogen and photosynthate that are mobilized and translocated from vegetative tissues to reproductive tissues. At vegetative and flowering stages, the percentage of mycorrhizal colonization was higher in roots irrigated with wastewater than those roots irrigated with Nile water. However, at the fruiting stage, the mycorrhizal colonization percentage in roots irrigated with Nile water was higher than that percentage in roots irrigated with wastewater (Fig. 3 A and B). It has been suggested that AMF are beneficial to plant nutrient uptake especially to P uptake (Smith and Read, 1997) and have better tolerance to HMs and might assist pioneering plant species to colonize metal contaminating sites (Zhang et al., 2006). Compared to the uninoculated control plants, the single inoculation with R showed an induction in mycorrhizal colonization of plants irrigated either with Nile water or wastewater. Xie et al. (1995) attributed this stimulatory effect to the Nod factors, the specific signal molecules of rhizobia that trigger the colonization and development of AMF via the so-called increased *nod* genes induction response. The results given in Fig. 4 (A and B) indicated that the co-inoculation with R+AMF in faba bean plants had a negative effect on nodulation number compared to the single inoculation with R only which showed the highest number of nodules in faba bean plants irrigated either with Nile water or wastewater at the different harvest stages. Meanwhile, single and dual inoculation with R and AMF significantly increased the nodulation number over uninoculated control treatment. Such findings concur with previous reports on common bean,

Table 4. Effect of R and AMF inoculants on soluble protein content (mg g^{-1} dw) of faba bean leaves irrigated with Nile water and wastewater at different growth stages. Means ± SD of triplicate measurements from two independent seasons are shown.

Total protein content (mg g^{-1} dw)						
Irrigation water used	Nile water			Wastewater		
Growth stage Treatment	Vegetative	Flowering	Fruiting	Vegetative	Flowering	Fruiting
Control	9.40 ± 0.09[d]	19.42 ± 0.11[d]	14.43 ± 0.092[c]	8.81 ± 0.11[c]	17.45 ± 0.13[d]	12.15 ± 0.12[c]
R	13.37 ± 0.27[b]	20.95 ± 0.14[b]	19.52 ± 0.08[a]	9.91 ± 0.20[b]	19.46 ± 0.12[b]	16.19 ± 0.14[a]
AMF	11.06 ± 0.10[c]	20.31 ± 0.12[c]	15.63 ± 0.18[b]	7.55 ± 0.15[d]	18.77 ± 0.15[c]	13.29 ± 0.08[b]
R+AMF	14.31 ± 0.12[a]	21.32 ± 0.12[a]	19.86 ± 0.38[a]	10.6 ± 0.03[a]	20.89 ± 0.24[a]	16.29 ± 0.04[a]

[a-d] means with different superscripts in the same column are considered statistically different (LSD test, P ≤ 0.05).
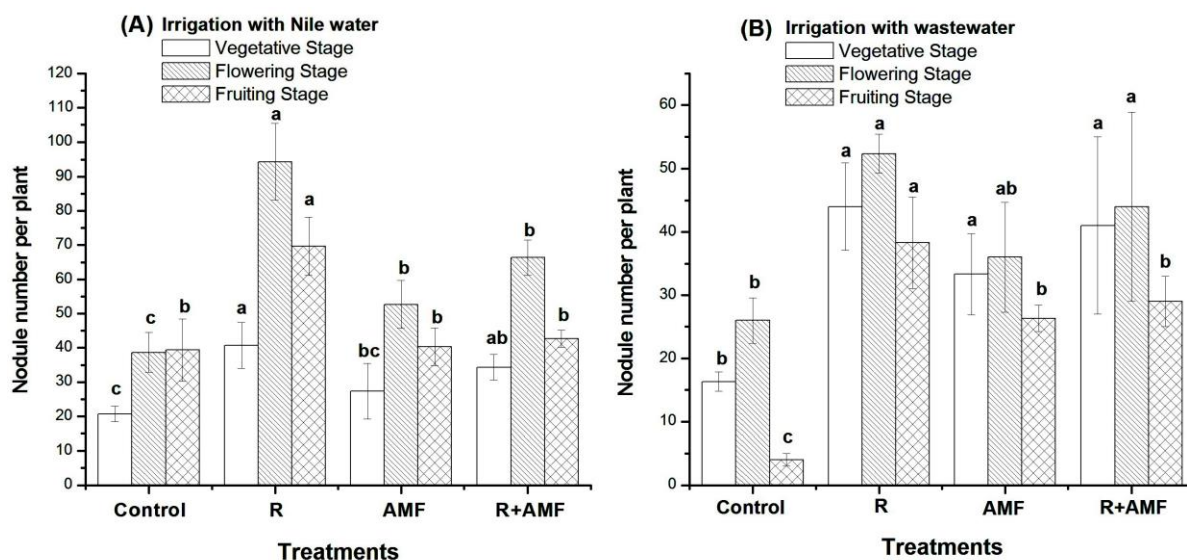

Fig 4. Effect of R and AMF inoculants on number of nodules per faba bean plant irrigated with Nile water (A) and wastewater (B) at different growth stages. Calculated mean is for 10 samples from two independent experiments ± SD. Different letters for each growth stage at different treatments are statistically different (LSD test, P ≤ 0.05).

which support that co-inoculation has a negative effect on the microsymbionts that are involved in this process (Bethlenfalvay et al., 1982), since nodulation and mycorrhizal colonization were higher when inoculated independently, and both parameters decreased with co-inoculation. AMF associated with legumes are an essential link for effective phosphorus nutrition, leading to enhanced nitrogen fixation and nodulation (Xavier and Germida, 2003). Our results further showed that at different harvest stages of faba bean plants irrigated with wastewater, a general decrease in the number of nodules per plant was obtained. This decrease is caused by HMs toxic effect on the growth of R (Bhandal et al., 1990).

HMs-binding capacity of R and AMF inoculants

In the present study, Cr and Mn were analyzed in the shoot and root systems of faba bean plants irrigated either with Nile water or wastewater using atomic absorption spectrophotometer. In particular, the wastewater irrigated plants showed extremely higher Cr and Mn concentrations than the Nile water irrigated plants and the root contained apparently higher concentrations of these HMs than the shoot (data not shown). Throughout the different plant growth stages, R and AMF inoculation either individually or in combination helped in lowering the HMs absorption by plants over the uninoculated control treatment. Roots of AMF inoculated plants were superior in HMs accumulation (data not shown). Mycorrhizal roots may act as a barrier against

metal transport from roots to the aerial part of the plant. This effect is attributed to metal adsorption on the hyphal walls, since chitin has an important metal-binding capacity (Joner et al., 2000). Additionally, glomalin, an insoluble glycoprotein produced by hyphae of AMF, was shown to bind potentially toxic elements including HMs (González-Chávez et al., 2004).

Materials and methods

Seeds

Seeds of faba bean (*Vicia faba* L. Giza 3) were obtained from the Legume Research Section, Field Crops Research Institute, Agricultural Research Center (ARC), Ministry of Agriculture, Giza, Egypt and selected for uniformity in size and color. The seeds were surface sterilized in sodium-hypochlorite (2% solution) then rinsed three times with distilled water.

Rhizobial bacteria and mycorrhizal fungi inoculants

The rhizobial inoculant used in this study was *Rhizobium leguminosarum* bv. *viceae*, obtained commercially from Biofertilization Unit, ARC, Ministry of Agriculture, Giza, Egypt. The bacterial strain was grown in 250 ml Erlenmeyer flasks containing 50 ml yeast mannitol broth (Somasegaran and Hoben, 1985) on rotating incubator shaker for 48 h at 35°C. For each plot, 100 g of the surface sterilized seeds

Table 5. Effect of R and AMF inoculants on PPO activity of faba bean leaves irrigated with Nile water and wastewater at different growth stages. Means ± SD of triplicate measurements from two independent seasons are shown.

PPO activity (U g^{-1} fw)				
Irrigation water used	Nile water		Wastewater	
Growth stage	Vegetative	Flowering	Vegetative	Flowering
Treatment				
Control	8.00 ± 2.00^b	8.00 ± 1.00^c	14.0 ± 2.00^c	10.0 ± 1.73^b
R	9.00 ± 2.00^b	10.0 ± 0.04^c	18.0 ± 1.73^{bc}	16.0 ± 5.29^b
AMF	10.0 ± 0.02^b	14.0 ± 2.00^b	20.0 ± 2.00^{ab}	34.0 ± 3.00^a
R+AMF	16.0 ± 1.73^a	20.0 ± 1.00^a	24.0 ± 3.00^a	40.0 ± 4.58^a

Units of PPO activity are expressed as changes in absorbance per minute (1U= 0.001 absorbance min^{-1}), [a-d] means with different superscripts in the same column are considered statistically different (LSD test, P $\leq$ 0.05).

were inoculated by 100 ml of the rhizobial suspension (each ml containing 10^8 cells). AMF were provided by Dr. Massoud, Soil, Water and Environment Research Institute, *Agric. Res. Center (ARC)*, Giza, Egypt. They included *Glomus mosseae*, *Gigaspora* sp., and *Acaulospora* sp. They were propagated in pots under onion roots in sterilized soil. After 60 days of growth, plant shoots were removed and the soil containing hyphae, spores and roots was air dried and used as inoculum. The inoculum was calculated using wet sieving and decanting technique (Gerdemann, 1955). The inoculated dosage for one seed was 3 g containing approximately 690 spores.

Irrigation water

Two types of water used for faba bean irrigation, Nile water and wastewater. The wastewater was obtained from Bahr El-Baqar drainage canal along Abu-Hammad, Sharkia, Egypt. The HMs concentrations in both water types were analyzed using atomic absorption spectrophotometer (Perkin Elmer, Inc. Norwalk, CT, USA) fitted with a specific lamp of particular metal using appropriate drift blanks. The analytical results showed that Nile water contained very low concentrations of Mn, Zn, Pb, and Cu (0.01 to 0.08 ppm) and some elements were almost not present as Cr (0.006 ppm), Ni (0.002 ppm), and Co (0.001 ppm). Cd was entirely absent. However, the wastewater contained elevated concentrations of Mn and Cr above the admissible limits for the irrigation purposes according to FAO (1985), other analyzed HMs were in the safe limits (Table 1).

Experimental design and planting

Surface sterilized faba bean seeds were planted during two successive growth seasons (first season: from November, 2010 to March, 2011; second season: from November, 2011 to March, 2012) at the greenhouse field of the Botany Department, Faculty of Science, Zagazig University. The range of mean temperature was 13±3ºC and that of relative humidity was 58 to 61%. The soil was classified as silty clay. It contained relatively higher proportion of silt, clay, and sand in a percent of 47, 43, and 20%, respectively. The soil paste revealed a relatively an alkaline pH of 7.70, E.C. of 1.0 ds m^{-1}, and a moisture content of 18.6%. Chemical analyses showed a higher percentage of K$^+$ (297 ppm). The available N and P were 19.8 and 8.20 ppm, respectively. The percent of organic matter content was 1.91% and that of CaCO$_3$ was 2.90%. Split plot design was followed in the present study. The area of each experimental plot was 6.25 m^2, including 4 rows 2.5 m long and 20 cm apart. The area was divided into two sections; the first section was irrigated with the Nile water, while the second section was irrigated with wastewater (HMs-polluted water). Each section was then divided into four plots. Each plot was 2.5 m length and 2.5 m width and

ridged into four rows. The seeds were sown individually each 10 cm within the row. In each section, the seeds of first plot were uninoculated (control plot). The second plot seeds were inoculated individually with R bacteria, the seeds of the third plot were inoculated individually with AMF and the seeds of the last plot were co-inoculated dually with R+AMF. The watering level of both irrigated sections was controlled using furrow type of irrigation that was conducted by small parallel channels along the section length in the direction of predominant slope, where water is applied to the top end of each furrow and flows down the section under the influence of gravity.

Sampling and physiological analyses

Plant samples (six replicates from each treatment) were harvested randomly from each plot at 45 days (vegetative stage), 75 days (flowering stage samples) and 120 days (fruiting stage samples). The plants were carefully uprooted with minimum disturbance of roots. The roots were washed with tap water. Roots and shoots were separated with the help of a sharp scissor. The samples were placed in an aluminum foil. Three samples were frozen at -20ºC for the required analyses. Other samples were shifted to an oven at 65ºC for 48 h till constant dry weight. The oven dried samples were taken out and ground to fine powder then stored.

Estimation of chlorophyll content

The photosynthetic pigments (chlorophyll a "Chl. a" and chlorophyll b "Chl. b") were estimated by the spectrophotometric method recommended by Metzner et al. (1965). A known weight (0.125 g) of the fresh young leaves was homogenized in 85% acetone for 5 minutes. The homogenate was centrifuged and the supernatant was made up to 30 ml with 85% acetone and measured spectrophotometrically against a blank of aqueous acetone at 644 and 663 nm. The concentration of the pigment fraction (Chl. a, Chl. b) was determined using reported equations (Metzner et al., 1965).

Estimation of total soluble protein content

Protein concentrations in the supernatant of finely powdered dried leaves tissues were determined by the method of Lowery et al. (1951) using bovine serum albumin as a standard.

Estimation of carbohydrate content

Soluble and insoluble sugars were estimated by the method of Naguib (1964). Briefly, powdered oven dried leaves (0.5 g) were extracted with 2% phenol water and 30% trichloroacetic acid. After filtration, the residue

(polysaccharides) was dried at 80ºC and the filtrate (mono and disaccharides) was hydrolyzed in 1N HCl then measured spectrophotometrically at 700 nm using Nelson's solution and glucose was used as standard. The insoluble sugars were determined by hydrolyzing of the dried residue in 1.5N H_2SO_4 under reflux condenser for 6 h. The solution was then cleared by lead acetate, deleaded by Na_2HPO_4 and its reducing value was determined using Nelson's solution, as mentioned above.

Extraction and estimation of IAA

Sample preparation for IAA estimation was performed according to Shindy and Smith (1975) with some modifications. Frozen fresh shoots (3 g per individual analysis) were ground in 80% (v/v) aqueous methanol, vacuum filtered, and re-extracted with methanol. The combined extracts were evaporated in a rotary and the aqueous phase was adjusted to pH 2.8 with 1% HCl and partitioned three times with equal volumes of ethyl acetate. The combined acidic ethyl acetate phase was reduced in volume to be used for determination of auxin which was measured colorimetrically at 530 nm using $FeCl_3$ reagent (Pilet and Chollet, 1970). IAA concentrations were expressed in $\mu g\ g^{-1}$ fresh weight using a standard curve.

PPO extraction and assay

PPO was extracted by homogenization of a known leaf fresh weight in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM sodium ascorbate, 1 mM EDTA and 0.5 M NaCl using an ice cold mortar (Gajewska and Sklodowska, 2008). PPO activities were then determined spectrophotometrically and expressed as changes in the optical density $min^{-1}\ g^{-1}$ fresh weight according to method described by Matta and Dimond (1963).

Determination of root nodules number and AMF root colonization

Immediately after each growth stage, the root system of ten plants from each plot was washed carefully with tap water to remove adhering soil particles. Nodules developed to a diameter of more than 0.5 mm were removed and counted. To estimate AMF root colonization, root samples were cut into 0.5 to 1 cm pieces, cleared, stained with 0.05% trypan blue, and observed with a light microscope to determine the percentage of root colonization (Zhang et al., 2006).

Statistical analyses

Results are expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using analysis of variance (ANOVA, SPSS software version 19) test followed by the least significant difference (LSD) test at 0.05 level.

Conclusions

Overall, this study demonstrated a comparative findings for the effect of irrigation with Nile water (clear water) and wastewater (HMs-polluted water) on some physiological and biochemical activities of faba bean plants inoculated individually, and in combination with R and AMF. Throughout the developmental stages of plant growth, findings showed that AMF, R and their combination may have a potential role in enhancement of total chlorophyll, soluble proteins, sugars and IAA contents. Regarding to the PPO activity, significant stimulatory effects have been arisen following faba bean dual inoculation with R and AMF. Therefore, this study suggested that co-inoculation with R and AMF may contribute to plant adaptive capacity enhancement and survival in HMs-polluted soils or wastewater-irrigated soils.

Acknowledgements

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