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ORIGINAL ARTICLES

Synergistic effect between *Pseudomonas putida* and arbuscular mycorrhizal fungi on *Vicia faba* L. growing in heavy metals polluted soil

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ABSTRACT

Field experiment was carried out to examine the interactions between *Ps. putida* and arbuscular mycorrhizal (AM) fungi and their effects on nodulation and some elements distribution in faba bean plants (Vicia faba L.) and soil in the presence of 25 or 50% mineral fertilizers (NPK). The soil before cultivation has noticeable amounts of copper and zinc in total form also Cu and Zn in available form. During the plant growth, the soil pollution rate increased with the investigated elements except Cu which decreased. The best treatment led to decrease the soil pollution of lead is to inoculation plants with AM fungi plus *Ps. putida* and in combination with 50% NPK. Stimulation of AM fungi colonization by a *Ps. putida* was noted. AM fungi alone significantly enhanced nodulation. Significantly more nodules had developed on the root systems of faba bean plants inoculation with *Ps. putida* + AM fungi compared to AM alone or uninoculated control. Plant inoculation with both organisms increased the population of *Pseudomonas* spp. The best treatments to decrease Cu pollution are those inoculated with AM fungi plus 25% NPK followed by AM fungi + *Ps.* +50%NPK in mature stage. All plant organs contained Pb in mature stage; the beast treatment to decrease Pb pollution is to use 25% NPK plus AM fungi. The concentration of zinc in all young plant parts is higher than 400 mg/kg then decreased when the plant growth reached mature stage. The use of 50% NPK plus AM fungi is the best treatment to decrease Zn content in the tested plant.

Key words: Arbuscular mycorrhizal (AM) fungi, faba bean plant, heavy metals, *Ps. putida*, and soil pollution

Introduction

Arbuscular mycorrhizal (AM) fungi is an integral part of the root system of most plants and constitutes an important group of organisms in soil microbial community (Ravnskov and Jakobsen 1999). The AM fungi play a key role in nutrients cycling by transport of nutrients, mainly phosphorus (P), from the soil to the plant. The fungal hyphae absorb P, but also other elements from the rhizosphere soils, and translocate them into the plant (Faber et al. 1990, Abdel Karem et al. 2000). AM-fungi were found to play an important role in heavy metal detoxification and the establishment of vegetation in strongly polluted areas (Turnau et al., 2010). However, only few studies have been conducted on the role of arbuscular mycorrhizae in soil polluted with heavy metals. It has been suggested that some mycorrhizal fungi could protect plants against the harmful effects of excessive heavy metals (Schuepp et al., 1987). Mycorrhizal colonization has been shown to be delayed, reduced, and even eliminated by high concentrations of Zn, Cu, Ni, and Cd (Leyval et al., 1991). Weissenhorn et al., (1993) isolated mycorrhizal fungi from two heavy metal polluted soils, which were found to be more resistant to Cd than a reference strain (Glomus mosseae).

Generally, the plant growth promotion due to inoculation with *Pseudomonas* may be attributed to the low molecular weight compound with a high affinity for Fe^{+3} produced by these organisms. In addition, many pseudomonads produce a number of antibiotics, which also have a role in biological control of soil-borne deceases (Dowling and O'Gara 1994, Thomashow and Macrodi 1997). In addition, some *Pseudomonas* spp. was found in sludge amended soils with increased levels of Cd (Bååth 1989).

Heavy metals ions in the parent soil material are set free in the process of soil formation in correspondence to the rate of weathering. The further fate of the ions depends on pedological factors such as pH, humus content, and redox potential as well as on external factors such as temperature, precipitations, erosion, land use practice etc. Accordingly, some elements are accumulated in the topsoil whereas others are leached out (Ernest 1991).

The purpose of the current experiment is to study the impacts of interactions between *Pseudomonas putida* and arbuscular mycorrhizal (AM) fungi on the distribution of heavy metals in organs of faba bean plant grown in polluted soil.

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Materials and Methods

A field experiment was set up at El-Fayoum Governorate to study the impact of dual inoculation of faba bean (*Vicia faba* L.) with *Ps. putida* in combination with arbuscular mycorrhizal fungi on the distribution of heavy metals in the different plant organs. The soil was a clay loam with pH of 7.9 (1:1, soil: water) and EC of 4.4 dS cm^{-1}. Soil nutrient determinations according to (Jackson, 1973) included 40.4 mg N kg^{-1}; 20 mg P kg^{-1} and 35 mg K kg^{-1}. Before cultivation, soil samples were taken from surface area (0-30 cm) to determine the soluble, available, and total elements in soil. The soluble elements in soil are extracted from a soil paste. Ammonium acetate-EDTA mixture (pH=4.65) and Aqua Regia were used to extract the available and total contents of the trace and heavy elements after Cottenie et al. (1982).

Seeds of faba bean variety "Rina Blanka" (provided from Dep. of Agronomy, Agricultural Research Center, Giza, Egypt) was sown at 30-cm spacing on rows (90 cm between them). The experiment was a factorial designed in a randomized complete block split plot with four replicates. Two levels of mineral fertilizer (50% or 25% of recommended mineral fertilizers) formed the main plot units. The sub-plot treatments were as follows: uninoculated (control), inoculated with AMF singly or in combination with *Ps. putida*. Each sub-plot (3x3 m) consisted of four rows of plants. The space between plots was 18 cm and between replications 1 m. Phosphorus in the form of calcium superphosphate (15.5% P_2O_5) and potassium in form of potassium sulphate were broadcasted and incorporated during soil tillage, while nitrogen fertilizer in form of ammonium sulphate was added in 2 equal doses after 15 and 30 days of sowing. Mycorrhizal inoculum consisted of onion roots, spores; hyphae and medium (peat: vermiculite: perlit 1:1:1) was used. Mycorrhizal inoculation was done by planting the seeds over a thin layer of mycorrhizal inoculum at the rate of 60g row^{-1} i.e. 100 kg/fed. *Pseudomonas putida* (obtained from culture collection of Agricultural Microbiology Dept., NRC, Giza, Egypt) inoculation was done by pelliting the seeds with a peat-based inoculant using gum Arabic.

After two months from cultivation and at harvest time, soil samples were collected from surface area to measure the available Zn, Cu and Pb. At both flowering and harvest times, plant samples were divided into roots, stem and leaves for the determination of Zn, Cu and Pb concentration into different plant organs. Trace elements were measured using Varian spectrAA220 atomic absorption spectrometer (AAS). Copper and zinc were determined in soil and plant applying micro-sampling technique. This could overcome the matrix- and nebulization difficulties in high salt sample solutions, while lead was determined applying graphite furnace-AAS. The percentage of root infection with AM fungi was evaluated using the magnified intersect method described by McGonigle et al. (1990). Mycorrhizal spores were counted according to Kormanik and McGraw (1982) and the number of spores was expressed to g dry $soil^{-1}$. Data were statistically analyzed using the SPSS (Statistical Package for the Sciences) system. All means were tested for significance using the methods described by Snedecar and Chochran (1980).

Results and Discussion

Table 1 shows the available and soluble concentrations of Zn, Cu, and Pb in surface layer of soil (0-30 cm) before cultivation. It is clear that the percentages of available to total elements (1.14% Zn, 8.48% Cu and 6.08% Pb) were lower. Also, the values of total and soluble copper, lead, and zinc are lower than maximum tolerable concentrations (B) and the concentrations in water-soluble (D), while total copper and zinc concentrations are higher than mentioned by Ewers (1991). In Egyptian soil the normal level of extractable Cu, Pb and Zn in non-polluted condition is ranged from 1.86 to 2.50 *u*g/g Cu, 1.17 to 1.61 *u*g/g Pb and from 1.56 to 2.64 *u*g/g Zn (DTPA extractable) (Aboulroos et al. 1996). However, Follet and Lindsay (1970) reported that the amounts higher than 1 and 0.2 *u*g/g are adequate for Zn and Cu in soil respectively. From the data obtained, according to Ewers 1991**,** Aboulroos et al. 1996)**,** it can be stated that the soil before cultivation was polluted with heavy metals.

Table 1: Element analysis of soil used before cultivation.

Element	Water soluble^A	Available^B
Cu	0.039	4.81
Pb	0.490	2.95
Zn	0.190	2.87

A: Soil paste extract, B: Extracted with NH_4OAC+EDTA.

Soil analysis, after cultivation showed that, the percentage of available Zn was increased in all treatments fertilized with 50% NPK than that of 25% NPK, except in case of dual inoculation with AM fungi and *Pseudomonas*. These results could be explained on the basis that colonization of root system with AM fungi increased the plant root volume in one hand, on the other hand AM hyphae collect and absorb higher amount of nutrients which present in the nearly soil, which intern increased their concentration in the rhizosphere soil. In addition, at the time of plant sampling, most elements remained in the soil which intern increased elements concentration in the rhizosphere soil. The available Cu was decreased in all treatments. However, the lowest percentage of copper was found in case of inoculation with the two biofertilizers in combination with 50%NPK. This could be deduced to the gradual increasing rate of nutrients uptake by developing plant growth.

After cultivation, available Pb in the soil was increased by increasing mineral fertilizers rate from 25% to 50% NPK. The highest percentage of available Pb was recorded in uninoculated treatment which is fertilized with 50% NPK. However, the lowest of Pb was in the treatment inoculated with AM fungi and *Ps. putida* in combination with 50% NPK (Fig 1).

From the data obtained, it can be concluded that the best treatment leads to dilute the harmful effect of heavy metals in soils was that inoculated with AM fungi + *Ps. putida* + 50% NPK. When soil fertilized with 25% NPK and inoculated with AM fungi, faba bean root length colonized by AM fungi was increased significantly from 50 to 75% by the inoculation with *Ps. putida*. At 50% mineral fertilizers, about 65% of the root systems of mycorrhizal plants inoculated with or without *Ps. putida* were mycorrhizal. Plants grown in soil uninoculated with AM fungi had low AM fungi infection. Increasing the mineral fertilizers rate from 25 to 50% inhibition mycorrhizal colonization (Fig 2). Stimulation of AM fungi colonization by a *Pseudomonas* spp. had been noted by Attia (1999), Barea et al. (1998), Meyer and Linderman (1986). The precise mechanism(s) that accounts for such microbial stimulation, however, has not been clearly identified yet. Most current evidence indicates that many microorganisms develop functions in the rhizosphere which may affect not only the plants but also other microbial members of the soil community (Kloepper et al. 1992). This is an effect exhibited by the so-called "mycorrhiza helper bacteria," which have a positive influence on formation of ectomycorrhizal and AM associations (Azcon-Aguilar and Barea 1992, Frey-Klett et al. 1997 and Garbaye 1994). Specialized activity, such as the production of vitamins, amino acids, hormones, etc., may be operating in microbe-microbe interactions involving AM fungi and *Pseudomonas* strains (Azcon-Aguilar and Barea 1995) and may account for the stimulatory effects found in this study.

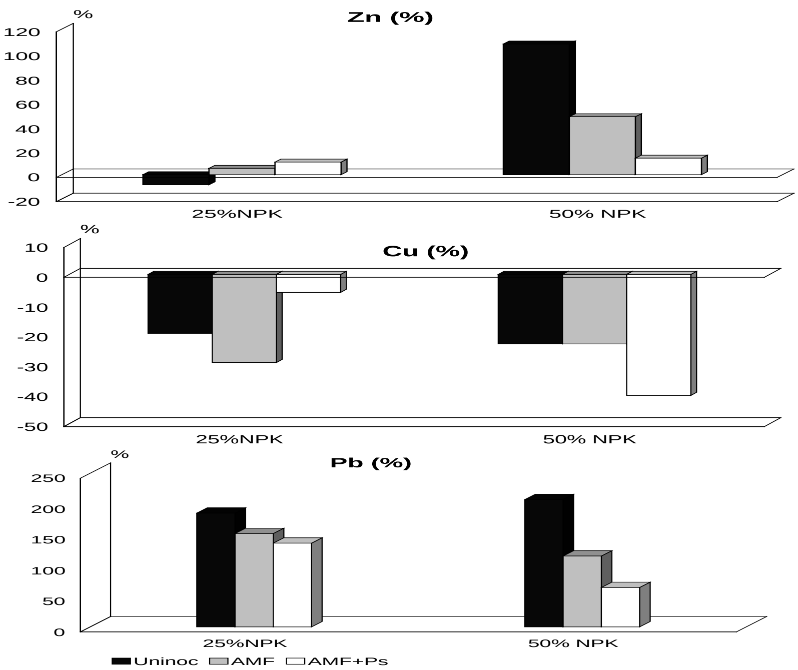

Fig. 1: The different available percentage of Zn, Cu and Pb in the treated soil.
*The different available of elements % = the concentration of elements in second time – the concentration of elements in first time / concentrations in soil before cultivation X 100

Biofertilizers inoculation significantly enhanced nodulation. However, significantly more nodules had developed on the root systems of faba bean plants inoculation with *Ps. putida* + AM fungi compared to AM alone or uninoculated control (Fig 2). The majority of the nodules of plants inoculated with AM fungi alone or in combination with *Ps. putida* appeared firm and pigmented at flowering stage. Nodules of control plants were noticeable smaller and less pigmented. Dually inoculated faba bean contained greater nodule dry weights than plants inoculated with AM fungi only or uninoculated plants (control). Our results confirm other studies in which *Rhizobium* nodulation was enhanced by mycorrhizal fungi and/or *Pseudomonas* spp. (Dashti et al. 1997, Dileep Kumar et al. 2001, Meyer and Linderman 1986 and Pacovsky et al. 1986). The mechanisms of growth and nitrogen fixation promotion by PGPR and AM fungi are not well understood; however, wide ranges of possibilities have been suggested, including both direct and indirect effects. The direct effects include an increase in mobilization of insoluble nutrients followed by enhancement of uptake by the plants (Lifshitz et al. 1987), production of antibiotics toxic to soil-born pathogens (Li and Alexander 1988), and production of plant growth regulators that stimulate plant growth. Indirect effects include positive effects on symbiotic nitrogen fixation by enhancement of root nodule number or mass (Grimes and Mount 1984, Zhang et al. 1996) and increased nitrogenase activity (Alagawadi and Gaur 1988).

Concerning, the population of *Pseudomonas* spp. in the rhizosphere soil of mycorrhizal plants, their number increased during the flowering stage from $3x10^4$ cfu g^{-1} dry rhizosphere soil in control plants to $4\text{-}5x10^4$ cfu g^{-1} dry soil. Plant inoculation with both organisms increased the population of *Pseudomonas* spp. to $6x10^6$ cfu g^{-1} dry soil. Since a large proportion of the photoassimilated carbon in plants is transported to the external mycorrhizal mycelium. These results could indicate that colonization of the root systemby mycorrhizal fungi may increase root exudates and thus indirectly affect bacterial growth in the rhizosphere. This conclusion could suggest that, the extraradical mycorrhizal hyphae can promote bacterial growth in the soil (Jakobsen and Rosendahl, 1990).

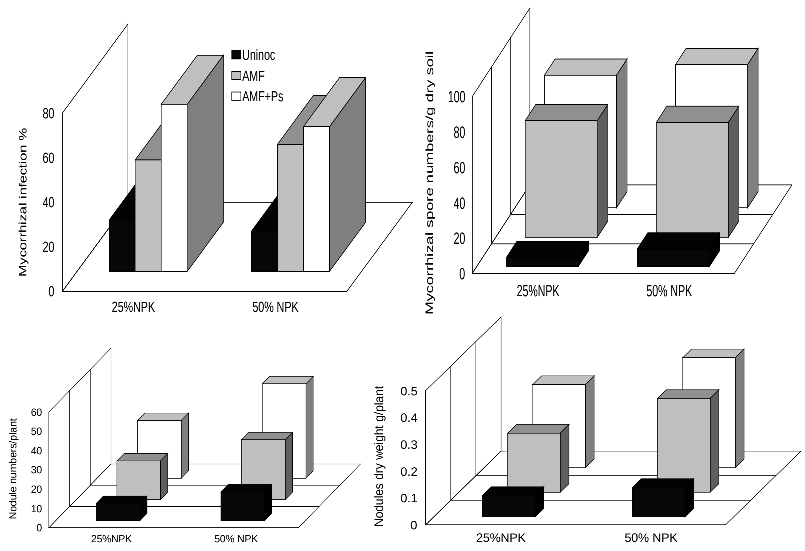

Fig. 2: Effects of interaction between AM fungi and *Ps. putida* on the mycorrhizal formation and nodulation of faba bean.

Zinc percentages in mature plant organs have been influenced by the dual inoculation with AM fungi and Ps. putida in combination with 25 or 50% of recommended NPK dose (Fig. 3). The previous mentioned figure indicated also that, application of the low rate of mineral fertilizers (25%), zinc concentrations were decreased in roots, stem, and leaves. On the other hand, increasing NPK rate up to 50% of the recommended dose increased percentage of Zn in the plant organs. Zn content was also found to be influenced by the plant age and vegetation status during the progressive growth period since the highest percentage of Zn concentration were recorded in young plants and tend to decrease us the plant has been aged. Fungal strains isolated from old zinc wastes also decrease heavy metal uptake by plants growing on metal rich substrates, limiting the risk of increasing the levels of these elements in the food chain (Ryszka and Turnau, 2007).

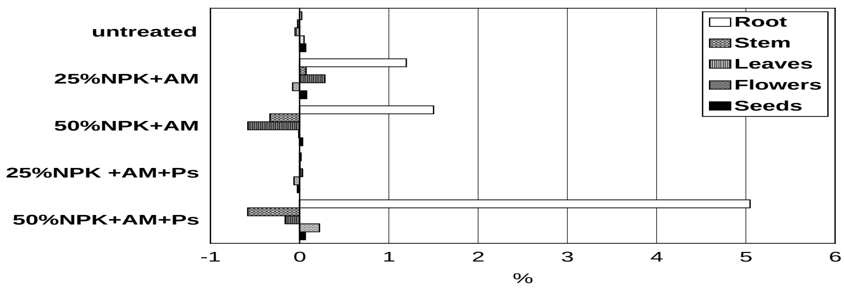

Fig. 3: Zinc distribution in the different organs of the tested plant inoculation with AM fungi and *Ps. putida* in addition to NPK.

Copper concentration increased in all organs of plant during plant growth except in stem at 50% NPK. Although, it was decreased in flowers and seeds in case of 50% NPK over those under 25% NPK. In case of inoculation faba bean with AM fungi + 25% NPK, copper concentrations decreased in stem and leaves with progress in plant growth (Fig. 4). Copper toxicity in plant is virtually unknown, a situation very much like that obtaining in man because of the protective mechanisms. Buck (1977) stated that regulatory mechanisms appear to limit the concentration of copper found in plant tissues to about 20 ppm. From the data obtained, it can be concluded that all organs of plant are polluted at the two stages of growth in case of use 25% NPK, as well as root and leaves at mature stage in case of 50% NPK, in uninoculated plants. All plant parts are polluted with copper in all treatment except the stem at the two stages of growth, seeds in case of fertilization with 50% NPK and inoculation with AM fungi and/or with *Ps. putida*. The best treatments are those inoculated with AM fungi + 25% NPK followed by AM fungi + Ps.+ 50% NPK. Mycorrhizal infection may increase Cu concentrations in the roots only (Kothari et al., 1991) or more in roots than in shoots (Killham and Firestone 1983). The mechanisms involved in this preferential Cu accumulation in mycorrhizal roots still unknown. Regarding to lead concentration it was decreased, mainly in leaves under 25% NPK and in stem under 50% NPK, while it was increased in flowers and seeds respectively.

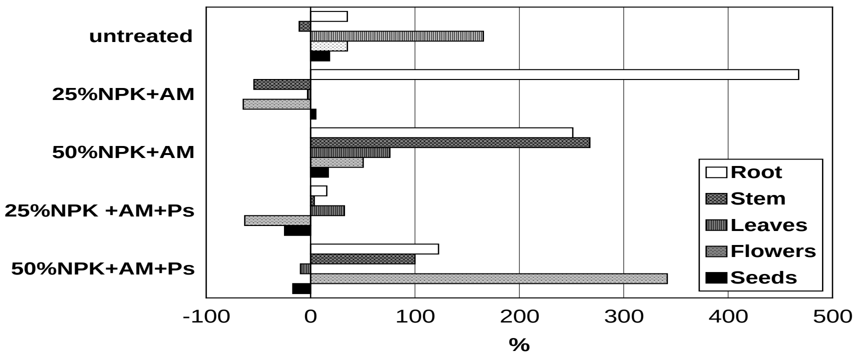

Fig. 4: Copper distribution within organs of plant inoculated with AM fungi and *Ps. putida* in addition to NPK.

Former, lead concentrations more decreased in all plant parts with plant growth when inoculated with AM fungi and *Pseudomonas* spp. (Fig. 5). In soils with natural lead concentrations (15-30 *u*g/g), only trace amounts of lead are absorbed by plants. The amount absorbed increased when the concentrations of lead in soil increases or when the binding capacity of soil for lead decreases at low organic fraction and low pH (Ernest 1991). It can be concluded that all plant parts are polluted with Pb according to National Academy of Science (1980). The best treatment is that of 25% NPK+ AM fungi in mature stage.

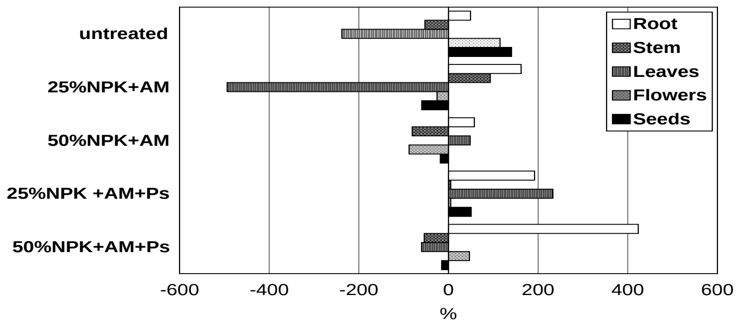

Fig. 5: Lead distribution within organs of plant inoculated with AM fungi and *Ps. putida* in addition to NPK.
*Different increasing or decreasing percentage of elements = concentration of elements in plant parts at second stage- concentrations in plant parts at first stage/ concentrations in plant parts uninoculated with AM fungi or AM fungi + *Ps. putida* X 100

AM fungi can enhance plant uptake of Zn and Cu, when these metals are present at low concentrations in soil (Kotharia et al. 1991). In contrast, several other authors (Leyval et al. 1991, Weissenhorn et al. 1995) reported that shoot concentrations of Zn, Cu, Pb and Cd decreased with AM colonization at high levels of available metals, whereas at lower levels metals uptake increased compared with nonmycorrhizal plants. The mechanisms behind the increasing the tolerance of mycorrhizal plants to heavy metal toxicity have not been elucidated (Bradley et al., 1982). The sequestration of metals by polyphosphate granules in fungal vacuoles was suggested as one possible mechanism (Turnau et al., 1993). It also was found that the cytoplasm of AM fungus contained more Cd, Ti and Ba than that of the host cells. Mycorrhizal plants inoculated with *Ps. putida* show pronounced vesicle development, this vesicle may be play a role in bind metals and reduced the translocation of metals to shoots. On the other hand, fungi can bind metals in the cell wall, thereby lowering the concentration of the metals in the soil solution.

The results of this field study indicate that metals-tolerant mycorrhizal inocula might be considered for reclamation of polluted soils and viability of plants and their metal resistance, particularly under adverse conditions.

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