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Cross Interaction of *Pseudomonas putida* and *Glomus intraradices* and its Effect on Wheat Root Colonization

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Abstract: To study the cross interaction of microorganisms in plant growth promotion and the effect of auxin in this interaction, *Pseudomonas putida* and *Glomus intraradices* were co-inoculated on wheat. For this purpose, a mutant that produced less amount of auxin was derived from the bacterial strain by chemical mutagenesis with ethyl methane sulfonate. Next both the wild-type strain and the mutant strain were labeled with *gusA* for a better detection on the roots. Finally, the bacteria were inoculated on wheat as single inoculants or combined with arbuscular mycorrhizal fungi. Controls included non-bacteria/non-arbuscular mycorrhizal fungi and arbuscular mycorrhizal fungi alone. Plants co-inoculated with the wild-type bacterial strain and *Glomus intraradices* had the highest stem and their fresh and dry shoot weight was more than other treatments. Also, it was observed that the wild-type strain significantly improved the mycorrhizal colonization which implies the beneficial effect of auxin on mycorrhizal colonization. Furthermore, *Glomus intraradices* increased the population density of the wild-type strain in the endorhizosphere. In the microscopic studies of plant roots, bacterial colonies were observed as blues lines on the surface and inside the roots as well as on the seed surfaces.

Key words: Plant growth-promotion, EMS, *gusA*, *Pseudomonas putida*, *Glomus intraradices*

INTRODUCTION

Almost all successful extant land plants in nature are associated with one or several Arbuscular Mycorrhizal (AM) fungi. Mycorrhizal formation has not been restricted to the Glomales but is also particular in Basidiomycota. In most cases, the basis of the mutualism is that the plant provides the major source of fixed carbon, whereas the fungus provides the host with mineral nutrients, water and in some cases protection from root pathogens (Boer *et al*., 2005). arbuscular mycorrhizal colonization affects the colonization pattern of roots by bacteria too, resulting in a greater spatial variability of bacterial distribution on AM roots (Christensen and Jakobsen, 1993). It can increase the population density of bacteria in the rhizosphere, have no effect on bacterial density, or decrease it (Abdel-Fatah and Mohamedin, 2000; Andrade *et al*., 1998; Bagyaraj and Menge, 1978; Medina *et al*., 2003; Van Aarle *et al*., 2003).

Bacteria able to colonize plant root systems and promote plant growth are referred to as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978). This group comprises, for example, the bacteria *Azotobacter*, *Azospirillum*, *Bacillus*, *Clostridium*, *Pseudomonas* and *Streptomyces*. The PGPR perform many important ecosystem processes. Besides participating in biological control of root pathogens, they also affect nutrient cycling and/or seedling establishment and soil quality (Vestberg *et al*., 2004; Barea *et al*., 2002). Of the above-mentioned bacteria, the fluorescent *Pseudomonas* is the one most extensively studied because of its role as a PGPR and biocontrol agent (Sastry *et al*., 2000; Vázquez *et al*., 2000). These bacteria significantly affect plant growth by increasing nutrient cycling, suppressing pathogens by producing antibiotics and siderophores or bacterial and fungal antagonistic substances and/or by producing biologically active substances such as auxins and other plant hormones (Khalid *et al*., 2004).

Different arbuscular mycorrhizal fungi species can differentially influence both the population and activity of pseudomonads (Marschner and Crowley, 1996) while on the other hand a significant effect of bacteria producing plant growth regulators on mycorrhizal development has been reported (Azcon *et al*., 1978). Knowledge of the interactions between soil bacteria and AM may be important for practical application of mycorrhizal inocula.

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Besides its use in the soil, bacterization (inoculation with bacterial cells) has been proposed for use along with inoculation with AM in artificial inert substrates (Liderman, 1992).

Auxins constitute a class of phytohormones that play important roles in the coordination of plant growth and development. Indole-3-acetic acid (IAA), the most abundant naturally occurring auxin, has been implicated in regulating a variety of developmental and cellular processes such as cell extension, cell division, vascular differentiation, root formation, apical dominance and tropisms (Napier and Venis, 1995). Besides plants, many soil and rhizosphere bacteria, including phytopathogenic, epiphytic and plant growth-stimulating bacteria, also produce IAA (Vande Broek *et al*., 1999).

Since on one side AMF hyphae could ameliorate the penetration of *Pseudomonas* into the host and its subsequent colonization and on the other side bacterial auxin production could effect AM colonization pattern and quantity by alteration in the morphology and development of hair roots, in the present study the emphasis was placed on the cross interaction of AM and an auxin producing *Pseudomonas.*

MATERIALS AND METHODS

Bacterial strains and growth conditions: This study was carried out in Iranian Soil and Water Research Institute from 2005 to 2007. *Pseudomonas putida* 53 was provided by microbial collection of Soil and Water Research Institute (Tehran, Iran) and was routinely grown at 28°C in King's medium B (KB) and stored at 4°C in KB. *Escherichia coli* S17-1 (λ pir) with the plasmid pCAM111::mTn5SSgusA11 was kindly provided by CAMBIA (Canberra, Australia) and cultured on Luria-Bertani (LB) medium, with spectinomycin for the maintenance of plasmid.

Bacterial mutagenesis: Bacterial mutants were obtained by EMS (Ethyl methane sulfonate) mutagenesis (Watson and Holloway, 1976). First, 10 μL of EMS (0.01%) was added to 1.5 mL of an exponential-phase culture in KB, shaked carefully and then incubated at 37°C for 1 h without shaking. One-millilitter aliquots of the treated culture was diluted 1 in 10 in KB and incubated at 37°C overnight with shaking. Next, a serial dilution of 10^{-1} to 10^{-5} was prepared and 0.1 mL aliquots of each dilution were cultured on KB plates. Finally, the plates were incubated at 26°C for 48 h. The mutant colonies were stored at 4°C in KB for further examination.

Quantification of auxin production: The mutants were propagated overnight in 5 mL of TSB (Tryptone Soya Broth) and then 20 μL aliquots were transferred in 5 mL of DF salts (Dworkin and Foster) supplemented with 500 μg mL^{-1} of $_{L}$-tryptophan. Following incubation at 28°C for 48 h, the bacterial cells were removed from the culture medium by centrifugation (9000×g, 5 min). Subsequently, 2 mL of Salkowski's reagent (1 mL of 0.5 M $FeCl_3$, 50 mL of 35% $HClO_4$) was added to a 1 mL aliquot of the supernatant and allowed to stand at the room temperature for 20 min before the absorbance at 530 nm was measured. Finally, the mutant that produced the least amount of auxin was chosen and designated as p36.

Labeling the *P. putida 53* and P36 with *gusA*: In the present study, *E.coli* S17.1, carrying pCAM111::mTn5SSgusA11, was used as the plasmid donor and *P. putida* 53 and p36 were used as the recipient strains. The *E.coli* strain was grown in LB supplemented with spectinomycin for the maintenance of plasmid. The *P. putida* strains were cultured on KB. After overnight incubation of the 3 bacterial strains at 28°C, the cells were harvested by centrifugation (6000×g, 5 min). Next, 100 μL of the donor strain and 100 μL of the recipient strains were mixed and cultured on KB. Following overnight incubation at 30°C, 2 mL of sterile 85% NaCl was spread on the KB plates and the recipient cells and the donor cells were mixed in it carefully. Then 1 mL aliquot of the NaCl containing the mixture of the cells was vortexed. Finally, a serial dilution of 10^{-1}-10^{-3} was prepared and plated onto selective medium containing spectinomycin and X-GlcA-cyclonexylammonium salt. The plates were incubated at 28°C for 72 h. The transconjugants appeared as blue colonies on the selective medium. After random measurement of auxin production of some of the isolates, two which produced the same amount of auxin as their wild forms were selected and designated as p53gusA and P36gusA.

Plant inoculation and cultivation: Wheat (*Triticum aestivum* L.) var. Zarin was used as the test plant. Surface sterilised seeds were pregerminated 24 h before use.The AM fungal species used was *Glomus intrarardices* belonging to the microbial collection of Soil and Water Research Institute (Tehran, Iran). The mycorrhizal inoculum consisted of sand, spores, hyphae and AM root fragments. An experiment with 6 treatments and 10 replications was conducted on wheat seeds as follow: 1. Control, 2. Inoculation with *Glomus intraradices,* 3. Inoculation with p53gusA strain, 4. Inoculation with P36gusA strain, 5. Inoculation with p53gusA strain and *Glomus intraradices*, 6. Inoculation with P36gusA strain and *Glomus intraradices*. For the mycorrhizal treatments,

0.25 g of the mycorrhizal inoculum and for the bacterial treatments, 0.5 mL of the bacterial inocula were added to the pots. Plants were grown for one month in a greenhouse under controlled conditions.

Harvest of the seedlings: After one month the seedlings were harvested following which the fresh and dry shoot weight as well as the stem height of the seedlings were measured.

Population densities of bacteria: First the population densities of the bacteria were measured in the rhizoplane. For this purpose, the roots were shaken free of excess sand. Then a serial dilution was prepared, plated onto selective medium and the plates were incubated at 28°C. After 48 h, the population densities were calculated by counting the blue colonies. Following surface sterilization of the roots by sodium hypochloride and subsequent mashing them, the previous procedure was used to calculate the population densities of the bacteria in the endorhizosphere.

GUS staining: First, the roots were shaken in 85% NaCl (50 mL) and 10% SDS (100 μL). Next, they were immersed in GUS assay solution composed of 50 mL of a phosphate buffer (19.5 mL of 0.2M NaH_2PO_4, 30.5 mL of 0.2 M Na_2HPO_4), 0.1 mL of 10% SDS and 0.5 g of X-GlcA and shaken overnight in the dark. Finally, they were studied by microscope.

Quantification of AM fungal colonization: The AM infection was assessed as described by Giovannetti and Mosse (1980). The roots were cleared in hot 10% KOH, treated with alkali H_2O_2 (3 mL of NH_4OH, 30 mL of 10% H_2O_2 and 567 mL distilled water) subsequently acidified in 1% HCl and in the end immersed in staining solution (876 mL of 90% Lactic acid, 64 mL of 90% Glycerol, 60 mL of distilled water and 0.1 g trypan blue. Each stained root sample was spread out on a square plastic Petri dish (10×10 cm) with a grid of lines on the bottom to form 0.5 cm squares. The presence or absence of infection was recorded through the microscope (magnification up to ×150) at each point where the root intersected a vertical or horizontal line. The calculation of infection is based on the formula of Newman (Giovannetti and Masse,1980).

RESULTS AND DISCUSSION

Mutagenesis with EMS (Ethyl Methane Sulfonate) resulted in the mutant P36 the IAA content of which was 1.43 lower than that of *P. putida* 53. Following successful labeling of P36 and *P. putida* 53 with *gusA*, blue colonies were observed on selective medium. Moreover, the studies showed that the insertion of the marker gene into the genome of *P. putida* 53 and p36 affected the growth characteristics of neither strains. Similar observation was made previously by Wang *et al*. (2004) on *Pseudomonas fluorescens* CS85.

Table 1: Effects of *Glomus intraradices* and the labeled bacterial strains on wheat growth parameters

Treatment	Shoot fresh wt. (g)	Shoot dry wt. (g)	Shoot height (cm)
Uninoculated control	1.5357b	0.35429c	42.586b
G. intraradices	2.0071a	0.48286b	44.071b
p53gusA	2.1343a	0.51143b	47.343a
P36gusA	1.9071ab	0.36571c	42.964b
p53gusA and *G. intrardices*	2.3214a	0.60143a	51.357a
P36gusA and *G. intrardices*	2.1286a	0.50571b	47.429ab

Means of shoot fresh weight with the same letter are not significantly different of LSD test ($p \leq 0.05$) and means of shoot dry weight and shoot height with the same letter are not significantly different of LSD test ($p \leq 0.01$)

According to Table 1, single treatments with P36gusA strain did not significantly affect any of the studied plant growth parameters and showed similar results to control treatments, while treatments with p53gusA strain did increase shoot height as well as both fresh and dry shoot weight in comparison to the control. This implies the importance of auxin production by *Pseudomonas* in plant growth promotion. According to Khalid *et al*. (2004), the more effective auxin producer PGPR had more promising effects on inoculated wheat seedlings. Furthermore, Jeon *et al*. (2003) demonstrated that strains of *Pseudomonas fluorescens* which produced auxin could promote the growth of two plants belonging to *Gramineae* family.

In this study, *G. intraradices* significantly augmented both fresh and dry shoot weight (Table 1). Mohammad *et al*. (2004) aslo suggested that the dry weight of the wheat plants inoculated with *G. intraradices* increased and the wheat plants produced more seeds per spike. As fungus provides the host with mineral nutrients, water and in some cases protection from root pathogens, this could result in better plant growth parameters.

Bacterial and mycorrhizal co-inoculated plants showed similar results to single mycorrhizal or bacterial treatments in terms of fresh shoot weight and shoot height but both of them significantly increased dry shoot weight. Plants co-inoculated with p53gusA and *Glomus intraradices* had the highest shoot and their fresh and dry shoot weight was more than other treatments. In the previous studies carried out by Vosatka *et al*. (1992) and Siddiqui and Mahmood (1998), the positive effect of co-inoculation with fluorescent Pseudomonads and Glomales on growth parameters of different plants have been reported. Furthermore, in the research carried out on

Table 2: Population densities of bacteria

Treatment	Rhizoplane	Endorhizosphere
	(CFU g^{-1})	
p53gusA	2.5×10^6	10^4
p36gusA	10^7	1.75×10^4
p53gusA and *G. intraradices*	2.3×10^6	3.38×10^5
p36gusA and *G. intraradices*	2.6×10^6	1.2×10^4

Table 3: Mycorrhizal colonization of wheat root

Treatment	AMF colonization (%)
G. intraradices	11.766b
G. intraradices and p53gusA	28.829a
G. intraradices and p36gusA	11.857b

Means with the same letter are not significantly different of LSD test ($p\leq0.01$)

3 different plants by Srivastava *et al.* (2007) the co-inoculation of AMF and pseudomonads proved to have better results. Also, Gamalero *et al.* (2004) demonstrated that *P. fluorescens* and *G. mosseae* when co-inoculated had a synergistic effect on tomato root fresh weight. and increased plant growth compared with singly inoculated plants.

According to Table 2, *Glomus intraradices* had no significant effect on the colonization of p53gusA strain in the rhizoplane but increased its population density in the endorhizosphere. As for P36gusA strain, *Glomus intraradices* had no significant effect on the population densities neither in the rhizoplane nor in the endorhizosphere. This could result from the effect of auxin on development of the root system and improving root elongation, therefore making it more possible for the mycorrhizal hypha to penetrate the root system and help bacteria colonize better in the endorhizosphere. Mansfeld-Giese *et al.* (2002) demonstrated that no significant difference in total bacterial number was observed between the mycorrhizal and non-mycorrhizal treatment of cucumber plants. Duponnois *et al.* (2005) did observe an increase in population densities of fluorescent Pseudomonads in *Acacia holosericea* inoculated with *G. intraradices* whereas Vázquez *et al.* (2000) and Marschner *et al.* (1997) observed a decrease in the population density of fluorescent pseudomonads in the presence of *Glomus mosseae.*

It was observed that p53gusA strain improved the mycorrhizal colonization and this confirms the beneficial effect of auxin on mycorrhizal colonization which could be the result of more developed root formation of plants and therefore easier penetration of mycorrhizal hyphae into the host roots (Table 3). Also, Niranjan *et al.* (2007) demonstrated that the exogenous application of IAA increased *Dalbergia sissoo* root colonization of *Glomus fasciculatum* by 60%. According to Gamalero *et al.* (2004) *P. fluorescens* when co-inoculated with *G. mosseae*, increased mycorrhizal colonization, whereas Roesti *et al.* (2006) proved that the percentage of wheat root colonization by AMF remained unaffected by the PGPR treatments.

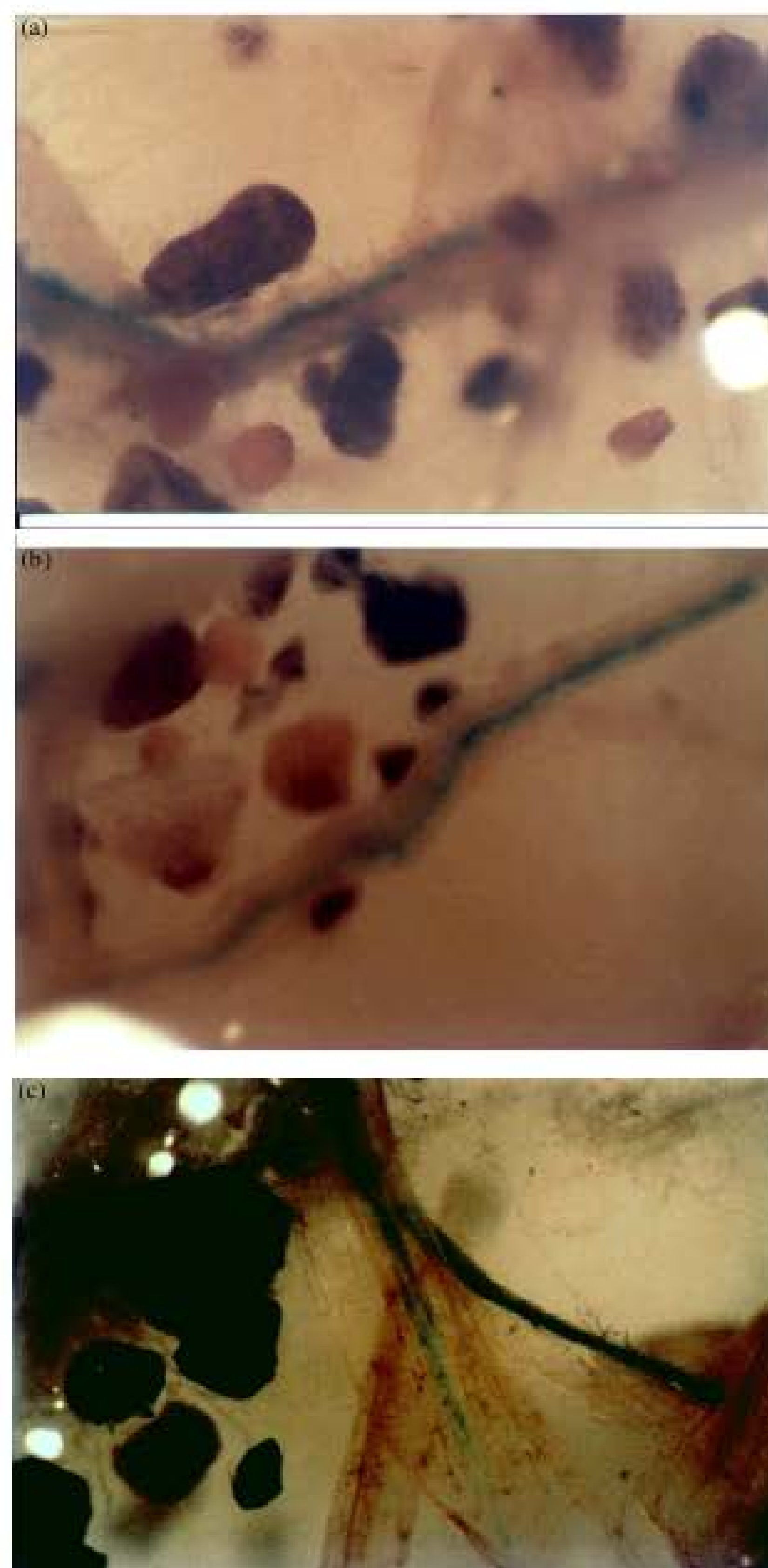

Fig. 1: Colonization of wheat roots by *P. putida* labeled with *gusA*

Microscopic studies followed GUS staining of the roots. Bacterial colonies were observed as blues lines on the surface and inside the roots as well as on the seed surfaces (Fig. 1a-c). Wang *et al.* (2004) used the same plasmid for *Pseudomonas fluorescens* on cotton roots and their results suggested that the labeled strains colonized all surfaces of the young plant root zones, such as roots hairs and lateral roots, although the distribution of the labeled strains on the root surfaces was not uniform. Similar to their observations, roots of uninoculated control plants did not have a blue zone which confirmed the lack of endogenous β-glucoronidase.

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