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The Biological Control Bacteria *Pseudomonas fluorescens* Inhibits Free Nitrogen Fixing Bacteria in the Rhizosphere

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A B S T R A C T

The present study was conducted in the laboratory and green house to explore the relation between *Pseudomonas fluorescens* as biocontrol agent and free N-fixers *Azotobacter chroococcum* and *Azospirillum lipoferium* bacteria and their effect on their population in rhizosphere, growth, N% in shoot and N content of wheat plants grown in sterilized and unsterilized sandy soil. Results revealed that *P. fluorescens* interacted negatively with the population of both nitrogen fixing bacteria in wheat rhizosphere and studied plant parameters. Results showed that decreased in *A. chroococcccum* counts in wheat rhizosphere of sterilized soil reached to 93.79% compared to *A. chroococcccum* count in single, while decrease was reached to 82.1 % in unsterilized soil. Also, data obtained showed that in sterilized soil; mixing of *P. fluoresences* with *A. lipoferum* caused highly significant lowering in population of *A. lipoferum* reached to 97.87 %, and by 91.53 % in unsterilized soil comparing with *A. lipoferum* inoculated singly. According to these results, it is necessary to separate these non-compatible types of bacteria, when added to the soil or seeds to avoid this antagonistic effect between biocontrol bacteria and introduced or indigenous microorganisms and select the compatible organisms in mixed biofertilizer inoculants.

Introduction

Microbial interactions in the rhizosphere are one of the main importance for plant growth and health Raaijmakers *et al*., (2009). Extensive studies were done for the beneficial effect of *fluorescent Pseudomonas* as plant growth promotion or biological control bacteria (Couillerot *et al*., 2009). Plant beneficial effects by *fluorescent pseudomonads* include the inhibition of soil pathogen fungi involving production of siderophores (Lemanceau *et al*., 1992) and antibiotic like substances secondary metabolites (Raaijmakers *et al*., 2002).

Nowadays, many commercial products for plant disease control are based on single bacteria strain like *Pseudomonas fluorescens* formulated to be used as biological control agents.

The use *Pseudomonas fluorescens* as PGPR and/or biological control agents requires the detailed understanding of its interactions with other bacteria and their performance in soil environment Gotz *et. al*., (2006). There are a few researches on the relation and interaction between microorganisms used as

inoculants. So, the aim of this study was to assess the interaction impact of *Pseudomonas fluorescens* bacteria when mixed with other rhizobacteria such as *A.chroococcum* and *A. lipoferum*, two of the most important species of plant-growth-promoting rhizobacteria and its effect on their rhizosphere population, growth, N% and content of wheat plants grown in sterilized and unsterilized sandy soil. Thus, selecting bacterial strain for production multiple inoculants and better understand of their interaction is an important consideration to produce efficient inoculum which promoting plant growth and/or suppressing diseases.

Materials and Methods

Microbial cultures used in the study

All strains of *Azotobacter chroococcum, Azospirillum lipoferum*, and *Pseudomonas fluorescens*, were locally isolated in previous study by (Abo-Baker, 2003). *Fusarium sp* pathogen fungi was supplied by Agriculture botany (microbiology) Dept., Fac. Agric., South Valley Univ., Egypt.

Test of antagonistic ability of *P. fluorescens* against *Fusarium sp* on plate agar media. (*in vitro*)

Dual culture technique (Dennis and Webster, 1971) was employed to prove (in vitro) the antagonistic ability of *Pseudomonas fluorescens* against the pathogenic fungi *Fusarium sp*. on potato dextrose agar (PDA) plates, (Beever and Bollard, 1970).

Study the interaction between nitrogen fixers and biological control of *P. fluorescences* in the rhizosphere of wheat plants (Pots experiments).

Pots (15 cm diameter) experiments were conducted using sterilized and unsterilized sandy soil to evaluate the interaction effect between nitrogen fixers (*A. chroococcum and A. lipoferum*) and biological control *Pseudomonas fluorescencs* on their population in wheat rhizosphere and determine the nitrogen fixing capacity by *A. chroococcum and A. lipoferum* as indicated by N% and N-content of wheat plants. The experimental soil has Sandy texture, pH 8.3, 3.25 $CaCO_3$ %, 1.95 dsm^1 EC, 0.011 Total N% and 20.6% water saturation capacity. Four grains of wheat (*Triticum aestivum* L. cv. Geza 168) / pot were planted and inoculated by adding 1 ml / grain of 6, 7 and 4 days old liquid culture of *A.lipoferum, A. chroococcum* and *P. fluorescens* contain $3x10^6$ CFU mL^{-1}, $5.4x10^5$ CFU mL^{-1} and 6.5 $x10^7$ CFU mL^{-1} respectively, the grains inoculated singly or in dual inoculation under sterilized and unsterilized soil.

Enumeration of selected microbial population in wheat rhizosphere

The soil samples were taken from rhizospheric zone of different treatments for enumerating the targeted microorganisms in soil using serial dilution method. For *pseudomonas* count, the poured plate count method was used, 1ml of the appropriate dilution was transferred to King's B. medium (King *et al*., 1954) agar plates which were incubated at 28°C for 3 days. *Pseudomonas* counts were recorded when particular colonies developed. The most probable number (MPN) method of (Alexander, 1965) was used for estimation of *Azotobacter* and *Azospirillum* population in wheat rhizosphere using Ashby's modified medium for *Azotobacter* (Abdel-Malek and Ishac, 1968) and semi-solid Nitrogen Free Bromothymol Blue (NFB) medium (Dobereiner *et al*., 1976) for *Azospirillum*. The MPN tubes were incubated at 28±2ºC for *Azotobacter* and 35ºC for *Azospirillum*. Microbial counts

were recorded when particular growth developed in tubes. The results were expressed as CFU g^{-1} dry soil.

Statistical analysis

All data obtained were analyzed using MSTAT- C (Russell, 1994) and one way analysis of variance was applied. The differences between means of the different treatments were compared using the least significant difference (L.S.D.) at 5% and 1% probability.

Results and Discussion

Antagonistic ability of *P. fluorescens* against *Fusarium sp.* on plate agar media (in vitro)

It is evident from Photo 1 that growth of the pathogenic fungi *Fusarium sp* was inhibited by *P. fluorescens in vitro* as indicated by the limited growth of fungi between *P. fluorescens* streaks comparing with the growth in control plate inoculated with fungi only. This can explained on the basis of the inhibitory effects of *P. fluorescens* on *Fusarium* pathogen fungi. In this respect Keel *et al*., (1990) and Thomashow and Weller (1995) reported that this inhibitory effect due to ability of *Pseudomonas fluorescens* to produce several metabolites with antibiotic or toxic activity which suppresses several pathogenic fungi.

Also, Nicodème *et al*., (2005) found that hydrogen cyanides, siderophores, and antibiotics was a metabolites produced by *Pseudomonas fluorescens* and consider the primary mechanism of biocontrol.

Interaction between nitrogen fixers and biological control agent *P. fluorescences* in the rhizosphere of wheat (Pot experiment)

The rhizosphere population of *A. chroococcum* single inoculation (0.145×10^3 CFU g^{-1}), *A. lipoferum* (4.032×10^3 CFU g^{-1}) and *P. fluorescens* (624.5×10^3 CFU g^{-1}) were significantly higher compared to uninoculated control in sterilized soil, while the counts in unsterilized soil reached to (0.134×10^3 CFU g^{-1}), (1.5×10^3 CFU g^{-1}) and (292.6×10^3 CFU g^{-1}), for *A. chroococcum*, *A. lipoferum* and *P. fluorescens*, respectively (Table 1).

A. chroococcum population in wheat rhizosphere as affected by mixing with *P. fluorescens*

Data of *A. chroococcum* count showed that under sterilized soil, mixing of *P. fluoresences* with *A. chrooccum* caused highly significant reduction in *A. chroococcum* population from 0.145×10^3 CFU g^{-1} to 0.009×10^3 CFU g^{-1} after 30 days; which reached to 93.79% comparing to *A. chroococccum* count individually inoculated (Table 1).

Also, data in (Table 1) showed that under unsterilized soil, highly significant reduction in *A. chroococcum* counts was happened when mixed with *Pseudomonas fluoresce* in wheat soil rhizosphere. This decrease was reached to 82. 1% comparing with single *A. chrooccum* treatments.

On the other hand, the decline in the number of *A. chroococcum* when mixed with *Pseudomonas fluoresce* was pronounced in sterilized soil compared to unsterilized one. In accordance with our results, Zehra *et al*., (2015) found that the efficiency of the biofertilizers was higher in case of application single beneficial microorganisms. They added that this might be due to the incompatibility of the rhizobacterial strains used as biofertilizers or due to antagonistic effect and secretion of some toxic substances by the bacteria.

A. lipoferum population in wheat rhizosphere as affected by mixing with *P. fluorescens*

Data in Table 1 showed that *A. lipoferum population* of wheat rhizosphere in sterilized and unsterilized soil influenced highly significant by mixing with *P.fluoresences.* Results showed that in sterilized soil; mixing *P. fluoresences* with *A. lipoferum* caused highly significant lowering in population of *A. lipoferum* reached to 97.87 %, comparing with *A. lipoferum* inoculation singly. Also, similar result was found in unsterilized soil where the numbers of *A. lipoferum* decreased by 91.53 % comparing with *A. lipoferum* singly inoculated. Olivier *et al*., (2011) found that *Pseudomonas fluorescens* produce antimicrobial metabolite 2,4-diacetylphloroglucinol production resulted in lower *Azospirillum* cell numbers per root system (based on colony counts) and restricted microscale root colonization of neighboring *Azospirillum* cells (based on confocal microscopy). They added; regardless of the *A. brasilense* strain used *pseudomonads* have the potential to interfere with *A. brasilense* phytostimulators on roots and with their plant growth promotion capacity.

Pseudomonas fluorescens population in rhizosphere as affected by both of *A. chroococcum* and *A. lipoferum* inoculation

Regarding the effect of both *A. chroococcum* and *A. lipoferum* on *P.fluoresences* count in rhizosphere of wheat, results showed significant decrease in the number of *P. fluoresences* when mixed with *A. chroococcum* and *A. lipoferum* reached to 22.34% and 28.6%, respectively under sterilized soil conditions, (Table 1). Furthermore, the decrease in the number of *P. fluoresences* under unsterilized soil was higher than in sterilized soil which reached to 68.73 % and 82,94% when mixed with *A. chrooccum* and *A. lipoferum*, respectively comparing with *P. fluoresences* single treatment solely, (Table1).

Results of Berendsen *et al*., (2012) revealed that, bacteria in rhizosphere may have direct beneficial or harmful effect to other soil bacteria or other microorganisms. Also, plant growth promotion rhizobacteria have developed mechanisms to affect and respond to each other. Combes-Meynet *et al*., 2011 found that production the 2,4-diacetylphloroglucinol (2,4-DAPG secondary metabolites from *P. fluorescens* may affect other PGPR like *Azospirillum*. The decrease in the number of *P. fluoresences* under both sterilized and unsterilized soils may be due to that *P. fluoresences* have low competence ability with other indigenous and introduce rhizosphere bacteria in unsterilized soil. inoculation with *P. fluorescens* alone was more effective than inoculations in which *P. fluorescens* was combined with other plant growth promotion rhizobacteria Anwar-ul-Haq *et al*., (2011). Competition for nutrients like organic acids, cell motility, space with other rhizobacteria is a factor that will negatively affect *P. fluorescens* population Prieto *et al*., (2011).

Plant dry weight (g/plant), N% and N-content (mg/plant) of wheat as affected by single and mixed inoculation

Results of wheat dry weight, N% and N-content showed that control treatment recorded the lowest values in selected wheat parameters whereas the wheat dry weight, N% and N-content in control treatment were higher in case of unsterilized soil than in sterilized for all treatments. This may be due to the activity of indigenous microorganisms in unsterilized soil to promote wheat plants growth. Also, results showed that all single inoculation treatments with the individually selected strains except *P. fluorescens* scored

highly significant increases in shoot dry weight, N% and N-content of wheat plants. The promotive treatments were those singly inoculated with selected *A. lipoferum* and *A. chroococcum* strains either in sterilized or unsterilized soil but the values were higher significantly in most parameters in sterilized soil than in unsterilized.

These results indicated that the promotive effects induced by the inoculated rhizobacterial strains is due to supplying plants with excess amount of fixed nitrogen, similar positive effects of wheat seed inoculation with *Azotobacter* were reported on tomato (Imam *et al.*, 1977).

Also, In addition, results showed that inoculation with *P. fluorescens*, either individually or co-inoculated in mixed culture with any of the tested strains did not generate significant improvement in shoot dry weight, N% and N-content. *P. fluorescens* strains are usually used in plant inoculation for biocontrol of pathogenic fungi diseases (Parke, 1990) and the promotive effects produced are mainly due to their antagonistic effects on the other deleterious microbes (Sorensen *et al*., 2001).

Inoculation of wheat grains with *P. fluorescens* in mixture with either *A. chroococcum* or with *A. lipoferum* significantly reduced wheat shoot dry weight, N% and nitrogen content comparing with *A. chroococcum* and *A. lipoferum* in single inoculation treatments. This significant reduction can be attributed to the inhibitory interaction between *P. fluorescens* and rhizobacterial inoculants (Abo–Baker, 2011). Moreover, the results showed that mixing *P. fluorescens* with *A. chroococcum* led to highly significant decrease in dry weight, N% and N- content of wheat plant comparing to *A. chroococcum* solely inoculated, the reduction in parameters values in unsterilized soil reached to 36.53%, 29.29% and 54.97% for shoot dry weight, N% and nitrogen content respectively, and 58.02%, 48.68% and 78.38 % in sterilized soil for the previous parameters, respectively.

Table.1 Effect of the interaction between biological control *P. fluorescens* on both of *A. lipoferum* and *A. chroococcum* population in wheat rhizosphere under sterilized and unsterilized soil

Inoculation Treatments	*A. chroococcum* Counts X 10^3		*P. fluorescens* Counts X 10^3	
	Unsterilized soil	Sterilized soil	Unsterilized soil	Sterilized soil
Uninoculated (control)	0.002±0.00	0.000±0.00	0.170±0.02	0.000±0.00
A. chroococcum single (S)	0.134±0.01	0.145±0.01	292.559±29.6	624.483±62.45
A. chroococcum+ *P. fluorescens* (Mixed)	0.024±0.002	0.009±0.0006	91.455±9.15	484.958±48.5
LSD 0.05	0.016		61.59	
LSD 0.01	0.023		86.34	
	A. lipoferum Counts X X 10^3		*P. fluorescens* Counts X 10^3	
	Unsterilized soil	Sterilized soil	Unsterilized soil	Sterilized soil
Uninoculated (control)	0.029±0.003	0.000±0.00	0.170±0.02	0.000±0.00
A. lipoferum single (S)	1.500±0.15	4.032±0.40	292.559±29.6	624.5±62.45
A. lipoferum + *P. fluorescens* (Mixed)	0.127±0.01	0.086±0.01	49.889±4.94	445.863±44.59
LSD 0.05	0.3132		59.57	
LSD 0.01	0.4391		83.76	

[Values are the mean of 4 replicates] ± shows standard deviation.

Table.2 Wheat dry weight, N% and N-content of wheat as affected by single and mixed inoculation with *A. chroococcum* and *P. fluorescens*

Inoculation Treatments	Dry weight (g/plant)	Shoot (N%)	N-content(mg/plant)
Sterilized soil			
Uninoculated (control)	0.290±0.03	0.052±0.01	0.151±0.03
P. fluorescens single (S)	0.352±0.03	0.061±0.01	0.215±0.05
A. chroococcum single (S)	0.717±0.07	0.152±0.01	1.096±0.22
P. fluorescens + *A. chroococcum* (Mixed).	0.301±0.03	0.078±0.01	0.237±0.05
Unsterilized soil			
Uninoculated (control)	0.363±0.04	0.065±0.01	0.236±0.05
P. fluorescens single (S)	0.409±0.04	0.077±0.01	0.315±0.07
A. chroococcum single (S)	0.594±0.06	0.140±0.01	0.835±0.17
P. fluorescens + *A. chroococcum* (Mixed).	0.377±0.04	0.099±0.01	0.376±0.07
LSD 0.05	0.075	0.015	0.1599
LSD 0.01	0.102	0.021	0.2167

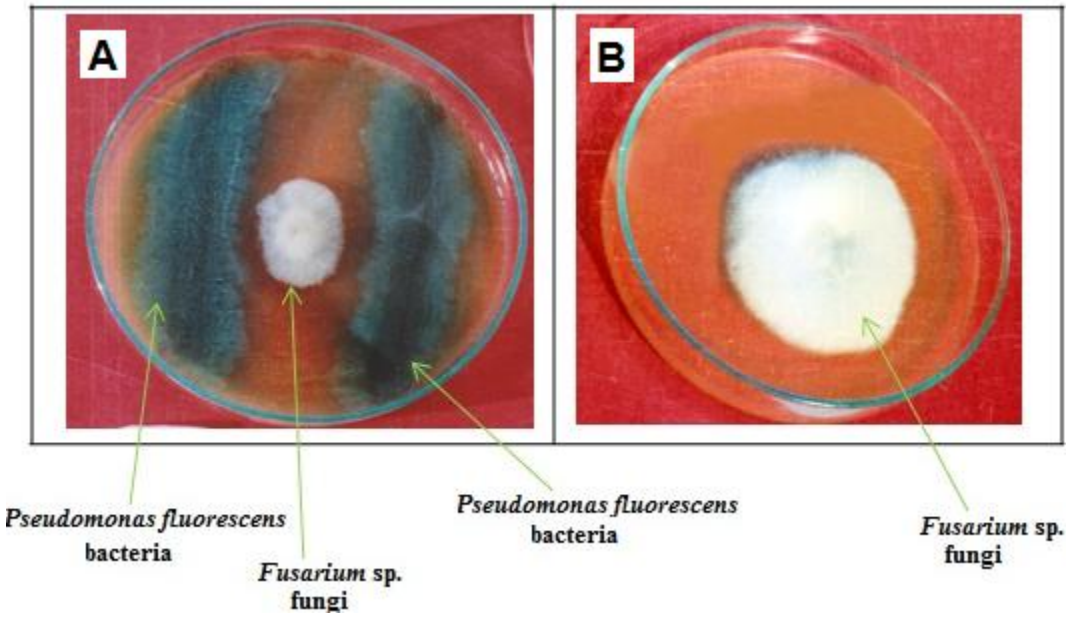
Values are the mean of 4 replicates; ± shows standard deviation

Table.3 Wheat dry weight, N% and N-content of wheat as affected by single and mixed inoculations with Azospirillum lipoferum and *P. fluorescens*

Inoculation Treatments	Dry weight (g/plant)	Shoot (N%)	N content mg/plant
Sterilized soil			
Uninoculated (control)	0.290±0.03	0.052±0.01	0.151±0.03
P. fluorescens single (S)	0.338±0.03	0.061±0.01	0.205±0.04
A. lipoferum single (S)	0.752±0.08	0.198±0.02	1.500±0.3
P. fluorescens + *Azospirillum* (Mixed).	0.336±0.03	0.063±0.01	0.212±0.04
Unsterilized soil			
Uninoculated (control)	0.363±0.04	0.065±0.01	0.237±0.05
P. fluorescens single (S)	0.374±0.04	0.067±0.01	0.253±0.05
A. lipoferum single (S)	0.594±0.06	0.156±0.02	0.934±0.19
P. fluorescens + *Azospirillum* (Mixed).	0.419±0.04	0.075±0.01	0.315±0.06
LSD 0.05	0.075	0.015	0.192
LSD 0.05	0.102	0.020	0.260

Values are the mean of 4 replicates; ± shows standard deviation

Photo.1 Antagonistic effect of *Pseudomonas fluorescens* against *Fuasrium* sp. on PDA medium (a) comparing with control (b).



It is obvious that, Cleary, this interaction that can take place in the rhizosphere between biocontrol agents and introduced or indigenous microorganisms need to explored in depth by more studies and researches to point out present and possible future trends in inoculants technology and they need be taken into account when developing bio-inoculants for crops to select the compatible organisms before recommending any mixed biofertilizer inoculants and to develop method and time of application.

In conclusion, these results indicate that, *P. fluorescens* as a biological control bacteria able to interfere with the performance of the other rhizosphere microbial like nitrogen fixers bacteria and interacted negatively. In this study the negative interaction effect was happened between biological control *P. fluorescens* and both of *A. chroococcum* or with *A. lipoferum* and influenced negatively their rhizosphere population as well as their N-fixing. On the other hands, because knowledge of rhizosphere bacterial interaction under field conditions is lacking; so we suggested that a compatibility test has to be done before recommending any multi biofertilizer inoculants and more researches under field conditions need to investigate the influence of biocontrol activity of bacterial inoculants and their effects on the indroduce or indigenous other microorganisms.

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