EFFECT OF THE CO-INOCULATION OF PLANT- GROWTH PROMOTING RHIZOBACTERIA AND RHIZOBIA ON DEVELOPMENT OF COMMON BEAN PLANTS (*Phaseolus vulgaris* L.)

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ABSTRACT

The objective of this study was evaluated the effect of two isolates of *Pseudomonas,* with low (ENA 4413) and high (ENA 4419) antagonistic effect to *R. leguminosarum* bv. *phaseoli* strains, on the development of common bean plants co-inoculated with a strain BR 10049. The experiment carried out in sand, showed that inoculation of common bean plants with only these rhizobacteria increased of root and leaf area as well as the total dry matter of the 20 days-old plants. Similar results were observed when common bean plants were co-inoculated with these two rhizobacteria and the strain of *Rhizobium* BR 10049. An increase of the number of nodules, dry mass of nodules and total dry mass was observed as compared with plants inoculated only with the strain BR 10049. However, there was a decrease of the nitrogenase activity with the co-inoculation of the rhizobacteria mainly with the isolate ENA 4419 that has shown high antagonistic effect to strain BR 10049. Nodules formed solely by rhizobia strains were pink while they appeared greenished when rhizobacteria were coinoculated. The presence of the rhizobacteria was confirmed by plate counting and fluorescence production when nodules were exposed to UV light. Light microscopy sections from nodules originated from the co-inoculation with rhizobacteria showed a small amount of infected cells. These cells were not fully occupied by the bacteroids and in addition they were not enclosed in a membrane envelope (peribacteroidal membrane). In general, the bacteroids appeared free in the host cell.

Key words: *Pseudomonas* sp, nodule structure, *R. leguminosarum*

INTRODUCTION

Fluorescent pseudomonads are considered as potential biocontrol agents of soilborne diseases (Weller, 1988). A mechanism often responsible for root disease suppression and potential control of soilborne plant pathogens appears to involve the production of antibiotic compounds (Fakhouri et al, 2001), siderophores (Sharma et al, 2003; Ams et al, 2002), producers of phytohormones (Kloepper et al, 1980) and biotic metabolites (Pedras et al, 2003).

In the case of legume plants, increments in dry matter or grain yield have been observed when rhizobacteria are co-inoculated with rhizobia strains on seeds (Grimes and Mount, 1984). Nevertheless, there are cases where no response or even negative effects have been observed (Polonenko et al., 1987). Since for practical applications, rhizobia and pseudomonads should be simultaneously inoculated, the interaction with both

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bacteria should be studied. De La Fuente et al (2002) showed antagonic activity against rhizobia *in vitro*, although the shoot dry weights of birdsfoot trefoil and rate of nodulation by rhizobia were not affected. Strains of pseudomonas produce the secondary metabolite, 2,4-diacetylphloroglucinol (DAPG) that is recognised as a key factor in the biocontrol of fungal diseases such as damping-off of sugarbeet (Fenton et al 1992; Loccoz-Moënne et al 1998) and antimicrobial metabolite like that, can cause some modifications on nodule occupancy and organization.

The objective of this study was evaluated the effect of two isolates of *Pseudomonas* with low (ENA 4413) and high (ENA 4419) antagonistic effect to *R. leguminosarum* bv. *phaseoli* strains on the development of bean plants co-inoculated with a strain BR 10049.

MATERIAL AND METHODS

1. Isolation and antagonistic tests

Forty two isolates of *Pseudomonas* with different degrees of fluorescent pigment production were isolated from the rhizosphere, rhizoplane or nodules of a common bean plants grown in the field of Rio de Janeiro State using the King B medium (King et al., 1954). These isolates were tested *in vitro* to evaluate the antagonist effect on 5 strains of *Rhizobium leguminosarum* bv. *phaseoli* using a double agar layer method. The rhizobial strains BR 10052, BR10028, BR10049, BR327 and DB1 were provided by the Culture Collection of Embrapa Agrobiologia.

The antagonistic test consisted in the inoculation of 48-hour-old cultures of *Pseudomonas* isolates on three points equidistant on the surface of plates containing 10 mL of King B medium. The inoculated plates were incubated for 48 h at 27°C followed by inactivation of bacterial growth with chloroform vapour treatment for 1 h. Afterwards, 5 mL of King B medium maintained at 45°C was inoculated with a rhizobial suspension of 10^8 ufc/mL and transferred to the surface of this *Pseudomonas* treated colonies. The plates were incubated for 48 h at 27°C and the halo of inhibition was measured. The experiment was established following a completely randomised design with 3 replications.

2. Root development of common bean plants inoculated with *Pseudomonas* isolates and grown in sand substrate.

Two isolates of *Pseudomonas* with high (ENA 4419) and low (ENA 4413) antagonistic effect to the *R. leguminosarum* bv. *phaseoli* strain BR10049 were selected and inoculated on seeds of common bean plants using the microbiolization method described by Luz (1983). The seeds of cultivar Carioca were previously surface sterilised with alcohol (70%) for 30 seconds followed by immersion in H_2O_2 (32%) for 1 minute and 30 seconds and ten times washing in sterile distilled water. Afterwards, the seeds were immersed in the rhizobacterial suspension for 2 h under agitation and sown in boxes containing sterile sand substrate maintained in greenhouse conditions. There were 6 lines/per box with 5 seeds per line. Five plants were harvested 20 days after sowing and the root area and total dry weight of the plant was determined.

3. Co-inoculation experiment

The fluorescent *Pseudomonas* isolates above (ENA 4419) and (ENA 4413) were co-inoculated with the *R. leg.* bv. *phaseoli* strain BR 10049. The seeds from the cultivar Carioca was surface sterilised as described above and sown in Leonard Jar (2 seeds/jar) containing sterile vermiculite: sand substrate (2:1 v/v) (Somasegaran & Hoben, 1985). Each seed was inoculated with the rhizobacterial and the rhizobial strain in the concentration of 10^8 ufc/mL. A completely randomised designed experiment was carried out in greenhouse conditions and the plants received a nutrient solution every two weeks. Plants were harvested at the grain filling stage (60 days) and determined the nodule number, nodule dry mass, nitrogenase activity and total dry weight. In addition, the green and pink nodules were harvested and used for microscopy analysis (Goi, 1993).

RESULTS AND DISCUSSION

Forty-two isolates of *Pseudomonas* sp producing fluorescent pigment with different intensity on King B medium were obtained from rhizoplane, rhizosphere and nodules of common bean plants (Martins et al., 2003). Two rhizobacteria isolates with high pigment intensity: ENA 4413 (from rhizoplane) and ENA 4419 (from nodule) were tested *in vitro* against 5 strains of *R. leguminosarum* bv. *phaseoli* to evaluate a possible inhibitory effect on growth of rhizobial strains. The results showed that the rhizobial isolate ENA 4419 produced a very high inhibitory effect on growth of all five rhizobia strains tested (Figure 1A). In contrast, the rhizobacteria ENA 4413 produced very low inhibitory effect or even no halo such as in the case of the rhizobia strain BR10049 (Figure 1A). An overview about the inhibitory effect of the rhizobacteria isolates on growth of rhizobial strains is shown in Figure 1B.

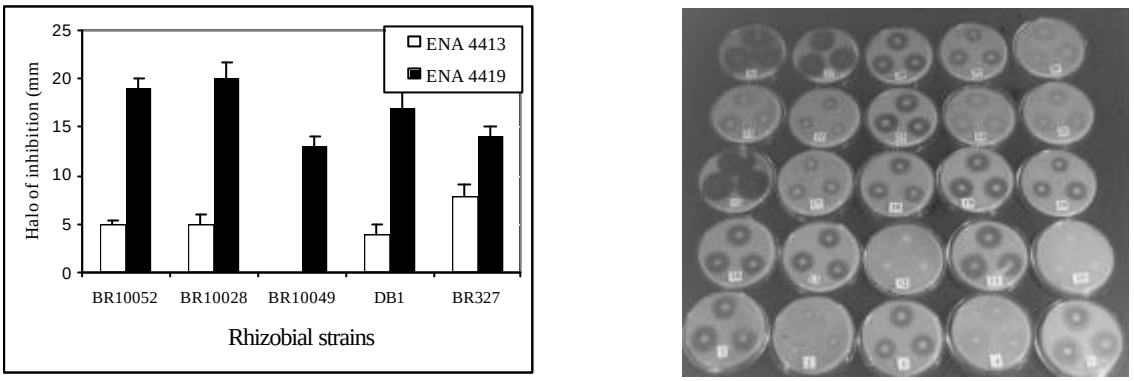

Figure 1. Antagonism of the *Pseudomonas* fluorescent isolates ENA 4413 and ENA 4419, isolated from rhizosphere and nodule of common bean plants, to *R. leguminosarum* bv. *phaseoli* strains: (A) Halo of growth inhibition (mm) of rhizobial strains BR 1052, BR 10028, BR 10049, DB1 and BR 327; (B) Examples of the antagonism to strains of *Rhizobium* as evaluated by the double agar layer method.

The ability of these two rhizobacteria isolates to stimulate root growth, leaf area and biomass accumulation of common bean plants grown in sand in the absence of rhizobia is presented in Figure 2A. The rhizobacteria ENA 4413 produced much higher increase of all parameters evaluated as compared to the isolate ENA 4419. In both cases, the rhizobacteria inoculation increased the root growth, leaf area and total dry matter as compared with the non-inoculated control (Figure 2A). An example of the effect of the rhizobacteria inoculation on the plant development is presented in Figure 2B.

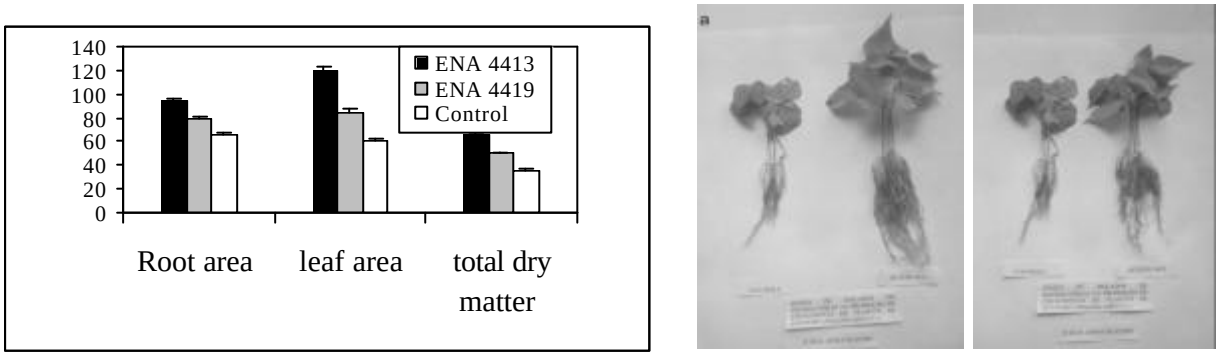

Figure 2. Effect of rhizobacterial inoculation of isolates ENA 4413 and ENA 4419 on growth of common bean plants grown in sand. (A) Effect on root area (mm^2/plant), leaf area (mm^2/plant) and total dry matter (cg/plant) production, (B) Photographs of common bean plants inoculated with isolates ENA 4413 and ENA 4419, respectively. Plants were harvested 20 days after planting.

The effect of the co-inoculation of rhizobacteria isolates and rhizobial strain BR10049 on common bean plants is shown on Table 1. There was an increase in the nodule numbers of plants co-inoculated with the rhizobacteria isolates and differed statistically from the plants inoculated only with the rhizobial strain (Table 1). The dry mass of the nodules also varied with the co-inoculation and, in contrast to the number of nodules, there was difference between the two-rhizobacteria isolates. The isolate ENA4413 induced much higher nodule mass and differed statistically from the isolate ENA 4419. Both rhizobacteria isolates induced higher nodule dry mass than the plants inoculated only with the strain of *Rhizobium*. However, the measurement of the nitrogenase activity of the nodulated plants showed much higher N_2-ase activity when inoculated only with *Rhizobium*, while the co-inoculation of the rhizobacteria isolate ENA 4419 reduced significantly the nitrogenase activity (Table 1). Nevertheless, the total dry matter accumulated in bean plants inoculated with the strain BR 10049 or co-inoculated with the isolate ENA 4419 did not differed statistically. The co-inoculation of ENA 4413 induced much higher total dry mass accumulation as compared with the other treatments (Table 1). In addition, the number of pods/plant was much higher in plants co-inoculated with this rhizobacteria and differed statically from the plants co-inoculated with ENA 4419 as well as from the plants inoculated only with BR 10049 (data not shown).

Table 1. Effect of co-inoculation of the rhizobacterial isolates ENA 4413 and ENA 4419 on common bean plants grown in the greenhouse and inoculated with *Rhizobium leg*. bv. *phaseoli* strain BR10049.

inoculant	Nº nodules/plants	Nodule dry mattermg/plant	Nitrogenase activity $nmolC_2H_4/h/plant$	Total dry matterg/plant
ENA 4413 + BR 10049	175a	130a	0,380b	3,70a
ENA 4419 + BR 10049	175a	115b	0,133c	3,03b
BR 10049	140b	105c	0,550a	3,10b
CV (%)	1,58	1,87	4,7	5,66

Plants were harvested 60days after planting.
CV – Coefficient of variation
Values in the column followed by same letter are not significantly different at P = 0.05 (Tukey)

Evaluation of these common bean plants inoculated only with *Rhizobium* strain BR10049 showed that the formed nodules were normal with pink colour, characteristic of the effectiveness of strain and nitrogen fixation ability. In contrast, nodule formed by plants co-inoculated with rhizobacteria isolates ENA 4413 and 4419 showed around 30% of nodules with a green colour inside and outside (data not shown). The presence of inoculated rhizobacteria isolates inside the nodules was confirmed by plate counting and fluorescence production when surface sterilised nodules were plated on King B medium and exposed to UV light, 24 hours after incubation. Acetylene reduction assays of individual green nodules showed nitrogenase activity lower than that of the pink nodules (data not shown) which is quite similar to that observed when the entire plant was evaluated (Table 1).

Light and transmission electron microscopy sections from nodules formed by the co-inoculation of rhizobial strain BR10049 with rhizobacteria isolates ENA 4419 showed abnormalities. Small amounts of infected cells (Figure 3B) were observed when compared with nodules formed by the plant inoculated only with the *Rhizobium* strain BR10049 (Fig. 3A). Infected cells showed lower population of symbiosomes when compared with infected cells of control nodules (Fig 4). Similar results were observed by Bolanos et al, (2003) in nodules from salt affected *Pisum sativum* plants. No peribacteroid membrane was identified. In general, the bacteroid appeared free in the host cell (Fig.5). No difference on nodule structure was observed when the *Pseudomonas* isolates selected for lower (ENA 4413) or higher (ENA 4419) antagonism to the *Rhizobium* strain BR10049 were compared.

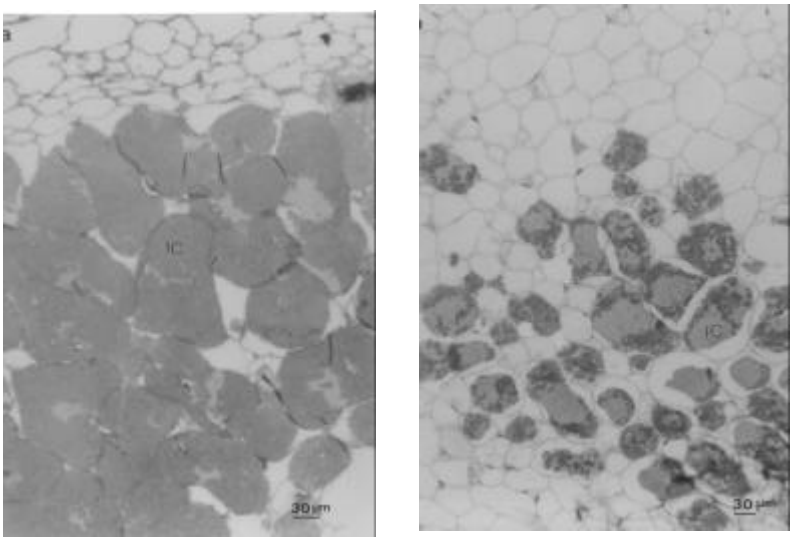

Figure. 3. (A) Photomicrograph of common bean nodule formed by inoculation solely of the *Rhizobium* strain BR10049 showing the infected cells (IC). (B) Photomicrograph of common bean nodule formed by *Rhizobium* strain BR10049, co-inoculated with the rhizobacteria ENA 4419 showing the infected cells (IC).

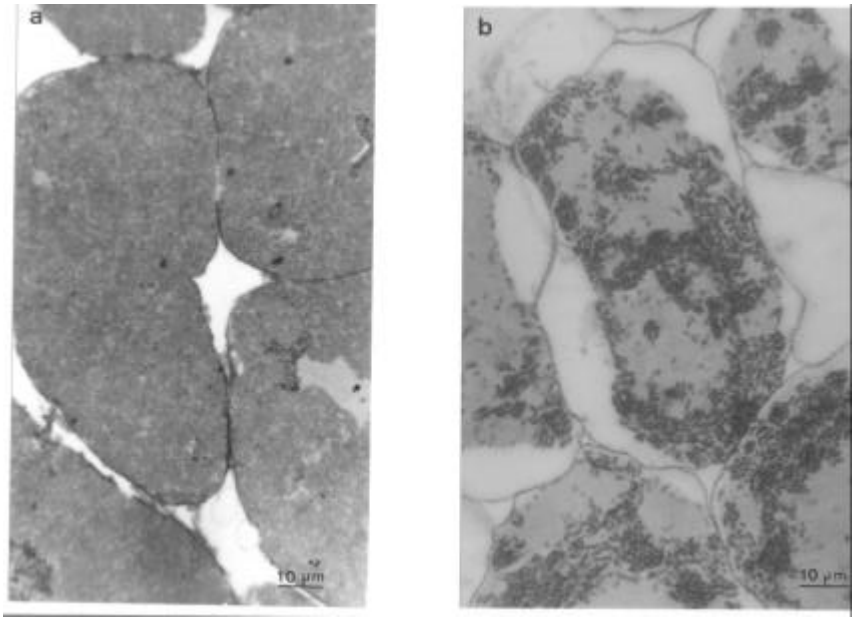

Figure 4. (A) Photomicrograph of common bean nodule formed by inoculation solely of the *Rhizobium* strain BR10049 showing the infected cells (IC). (B) Detail of the common bean nodule formed by the *Rhizobium* strain BR10049 co-inoculated with the rhizobacteria ENA 4419 showing that the infect cells (IC) were not fully occupied by the bacteroid.

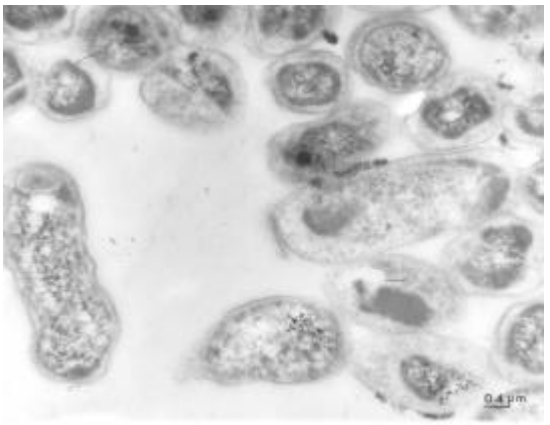

Figure 5. Electron micrograph of common bean nodule formed by the *Rhizobium* strain Br10049, co-inoculated with rhizobacteria ENA 4419 showing that the bacteroid (B) were not enclosed by the peribacteroid membrane.

It seems that the presence of *Pseudomonas* affected rhizobia multiplication and subsequent nodule occupancy during the process of nodule formation. Fenton et al (1992) suggested that the production of antagonistic substances is responsible for this inhibition effect. In this study, the green coloured nodules formed by the symbiosis *Rhizobium*/legume plants may be caused by the presence of fluorescent rhizobacteria co-inoculated with the strain of *Rhizobium*. Plant nodule cells of *Trifolium pratense* were not colonizes by *P. fluorescens* (Marek-Kozaczuk et al 2000) which colonized

Many authors have studied the practice of co-inoculation of legumes with plant-growth rhizobacteria (Bagnasco et al , 1998; Marek-Kozaczuk et al., 2000; DE LA Fuente et al, 2002; Martins et al, 2003; Lucas Guarcia et al., 2004). In many case, no effect or even negative effect on plant grown and rhizobia symbiosis have been reported. In this study, it was observed that the co-inoculation of

the rhizobacteria with the *Rhizobium* strain BR 10049 affected mainly the nitrogenase activity of the nodules. This effect was much more pronounced with the isolate ENA 4419 (antagonic rhizobacteria) as compared with the isolate ENA 4413 (no antagonic effect). However, it did not reflect in reduction of nodule numbers, nodule dry mass and total dry matter. Indeed, the co-inoculation increased the agronomic parameters evaluated mainly with the isolate ENA 4413. Similar results were observed when the common bean plants were grown in sand and inoculated with these rhizobacterial isolates. The lower nitrogenase activity of the co-inoculated plants may be due to the co-occupation of the nodules by the rhizobacteria isolates that could be draining part of the carbon source for its maintenance. Other authors have reported the effect of inoculation sequence that is determinant in the positive or negative effect on the symbiosis (Lucas Garcia et al. 2004). In this study, the effects of co-inoculation on plant growth were positive and indicate that rhizobacterial strains with activity antagonic to *Rhizobium* should be avoided. Additional field studies should be carried out to determine the behaviour of these rhizobacterial isolates co-inoculated with *Rhizobium* in common bean plants.

REFERENCES

AMS, D. A.; MAURICE, P. A.; HERSMAN, L. E.; FORSYTHE, J. H. Siderophore production by an aerobic *Pseudomonas mendocina* bacterium in the presence of kaolinite. **Chemical Geology**, V. 188, n. 3-4, p.161-170, 2002.

BAGNASCO, P.; DE LA FUENTE, L.; GUALTIERI, G.; NOYA, F.; ARIAS, A. Fluorescent pseudomonas spp. As biocontrol agents against forage legume root pathogenic fungi. **Soil Biology and Biochemistry**, V. 30, p.1317-1322, 1998.

DE LA FUENTE, L.; QUAGLIOTTO, L.; N.; FABIANO, E.; ALTIER, N.; ARIAS, A. Inoculation with *Pseudomonas fluorescens* biocontrol strains does not affect the symbiosis between rhizobia and forage legumes. **Soil Biology & Biochemistry**, V. 34, p.545-548.

FAKHOURI, W.; WALKER, F.; VOGLER, B.; ARMBRUSTER, W.; BUCHENAUER, H. Isolation and identification of N-mercapto-4-formmylcarbostyril, an antibiotic produced by *Pseudomonas fluorescens*. **Phytochemistry**, V. 58, n. 8, p.1297-1303, 2001.

FENTON, A. M.; STEPHENS, P. M.; CROWLWY, J.; CALLAGHAN, M.; O' GARA, F. Exploitation of gene (s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a Pseudomonas strain. **Applied Environmental Microbiology**, V. 58, p.3873-3878,1992.

GOI, S.R. **Nitrogen Nutrition of Woody Legumes**. 1993. 199p. PhD thesis. University of Dundee. Dundee, Scotland.

GRIMES, H.D. AND MOUNT, M.S. 1984. Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. **Soil Biology and Biochemistry**, V. p. 16:27-39, 1984.

KING, E. O.; WARD, M. K.; RANEY, O. E. two simple media for the demonstration of pyocinin and fluorescein. **Journal of Laboratorial Clinical Medicine Veterinary**, V. 44, p.301-307, 1954.

KLOEPPER, J.W., LEONS, J., TEINZE, M. AND SHROTH, M.N. Ps*eudomonas* siderophores: a mechanism explaining disease supressive soil. **Current Microbiology**, V. 4, p.317-320, 1980.

LOCCOZ-MOËNNE, Y.; POWELL, J.; HIGGINS, P.; McCARTHY, J.; O' GARA, F. An investigation of the impact of biocontrol *Pseudomonas fluorescens* F 113 on the growth of sugarbeet and the performance of subsequent clover-*Rhizobium* symbioses. **Applied Soil Ecology**, V. 7, p.225-237, 1998.

LUCAS GUARCIA, J.A.; PROBANZA, A. ; RAMOS, B. ;BARRIUSO, J.; GUTIERREZ, M. Effects of inoculation with plant growth promoting rhizobacteria (PGPRs) and *Sinorhizobium fredii* on biological nitrogen fixation, nodulation and growth of *Glycine max* cv. Osumi. **Plant and Soil**, V. 267, 143-153, 2004.

LUZ, W. C. da. Microbiolização de sementes para o controle de doenças de plantas. In: Luz, W. C. da; Fernandes, J. M.; Prestes, A.M.; E. C. Picinini (eds). **Revisão Anual de Patologia de Plantas (RAPP)** V. 1, p.37-77, 1993.

MAREK-KOZACZUK, M.; KOPCIDSKA, J.; LOTOCKA, B.;GOLINOWSKI,W.;SKORUPSKA, A. Infection of clover by plant growth promoting *Pseudomonas fluorescens* strain 267 and *Rhizobium leguminosarum* bv. *trifolii* studied by mTn5-*gus*A. **Antonie von Leeuwenhock** V. 78, p. 1-11, 2000.

MARTINS, A.; KIMURA, O.; RIBEIRO, R.L.D.; BALDANI, J. I. Efeito da microbiolização de sementes com rizobactérias fluorescentes do gênero *Pseudomonas* sobre a "murcha

fusariana" do feijoeiro (*Phaseolus vulgaris* L.). **Agronomia**, V. 37, n.1, p.69-75, 2003.

PEDRAS, M. S.C.; ISNMAIL, N.; QUAIL, W.; BOYETCHKO, S. M. Structure, chemistry, and biological activity of pseudophomins A and B, new cyclic lipodepsipeptides isolated from the biocontrol bacterium. **Phytochemistry**, V. 62, n. 7, p. 1105-1114, 2003.

PLONENKO, D.R., SCHER, F.M., KLOEPPER, J.W., SINGLETON, C. A . LALIBERTÉ, M. AND ZALESKA, I. Effects of root colonizing bacteria on nodulation of soybean roots of *Bradyrhizobium japonicum.* **Canadian Journal of Microbiology**, V. 33, p.498-503, 1987.

SHARMA, A.; JOHRI, B. N.; SHARMA, A. K.; GLICK, B. R. PLANT GROWTH-PROMOTING BACTERIUM *Pseudomonas* sp. strain GRP3 influences iron acquisition in mung bean (Vigna radiata L. Wilzeck). **Soil Biology & Biochemistry**, V. 35, p. 887-894, 2003.

SOMASEGARAN, P.; HOBEN, H. J. **Methods in Legume-Rhizobium Tecnology**. University of Hawaii, NIFTAL, 365p. 1985.

WELLER, D.M. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. **Annual Review of Phytopathology**, v.26, p.379-407, 1988.