

Full Length Research Paper

Antagonistic bioactivity of an endophytic bacterium H-6

Ya Wang¹, Qing-gui Zeng¹, Zhi-bin Zhang¹, Ri-ming Yan¹ and Du Zhu^{1,2*}

¹Key Lab of Protection and Utilization of Subtropic Plant Resources, Jiangxi Normal University, Nanchang 330022, People's Republic of China.

²Yichun University, Yichun, Jiangxi 336000, People's Republic of China.

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An endophytic bacterium, H-6, was isolated from leaves of *Huperzia serrata* grown in the Lushan Mountain, China. The strain was identified as *Burkholderia* sp. H-6 based on morphological, physiological and biochemical methods as well as on 16S rDNA analysis. This strain inhibited mycelium growth *in vitro* of 6 plant pathogenic fungi, especially of *Phytophthora capsici*, *Fusarium graminearum* and *Sclerotinia libertiana*. In greenhouse pot experiments, soil drenches with cell densities of 10^6 , 10^8 and 10^{10} CFU ml⁻¹ H-6 reduced significantly *P. capsici*, in pepper seedling by 51.7, 58.7 and 60.2%, respectively, compared to the inoculated control, 3 weeks after sowing. Growth parameters such as lengths and fresh weights of roots and shoots of *P. capsici*-inoculated control plants were significantly lower compared to *P. capsici*-inoculated and H-6-treated plants, which is an added advantage of the strain used as potential biocontrol agent in future.

Key words: Endophytic bacterium, 16S rDNA gene, antagonistic activity, *Huperzia serrata*.

INTRODUCTION

Soil-borne diseases are a serious concern in the world agriculture. So far, many phytopathogenic fungi, such as *Phytophthora capsici* and *Sclerotinia sclerotiorum* have been reported as the causal agents of soil-borne diseases and cause significant loss to growers (Lee et al., 2008; Rocha et al., 2009). Even though chemical inputs such as pesticides showed promising results in controlling the disease, phytotoxicity and chemical residues may pose a serious threat to the environment and human health (Rajkumar et al., 2005).

Biological control has been described as a non-hazardous strategy to reduce crop damage caused by plant pathogens when compared to the exclusive use of the chemical control of plant diseases (Bagnasco et al., 1998). The mechanisms of biocontrol agents can antagonize soil-borne pathogens are generally included predation, competition for ecological niches and nutrients, antibiosis and induction of a plant defence response (Thomashow and Weller, 1995; Sturz et al., 2000; Kong and Ding, 2001; He et al., 2009). In this context, biological control appears to be a rational alternative for agriculture.

Recently, there has been considerable interest in developing biological strategies using endophytic bacteria with antimicrobial activities for controlling pathogens (Hallman et al., 1997; Kavino et al., 2007). Endophytic bacteria living in the healthy tissues of plants are relatively unstudied and potential sources of novel natural products for exploitation in agriculture, medicine, and other industries (Strobel and Daisy, 2003). In fact, some strains belonging to the genus *Alcaligenes*, *Kluyvera*, *Burkholderia* and *Bacillus* are effective biological control agents (Assis et al., 1998; Bevivino et al., 2000; Kudryashava et al., 2005). Therefore, searching for new endophytic bacteria is a way of controlling plant diseases by biological control methods.

In this paper, an isolate of endophytic bacterium from *Huperzia serrata* was screened for its antimicrobial activity *in vitro*. The dual culture tests revealed that this endophytic bacterium strain displayed a wide-spectrum antimicrobial activity against 6 phytopathogens fungi. It especially strongly inhibited the growth of *P. capsici*, *Fusarium graminearum* and *Sclerotinia libertiana*. Also in the greenhouse pot experiments the strain was proved to be very efficient in biological control of *P. capsici*. Finally, the endophytic bacterium strain H-6 was identified through morphology, physiological and chemical characteristics and homology of 16S rDNA gene sequence by NCBI program

*Corresponding author. E-mail: zhudu12@163.com. Tel: +86 795 3200 616.

BLAST and phylogenetic tree analysis.

MATERIALS AND METHODS

Test phytopathogens and media

Six strains of fungal for antimicrobial activities assays were used in this study. The tested isolates: *S. sclerotiorum*, *F. graminearum*, *S. libertiana*, *P. capsici*, *Sesame fusarium wilt* and *Rhizoctonia solani* were all stored in our laboratory. Potato dextrose agar (diced potatoes, 200 g/l; dextrose, 20 g/l; agar, 18 g/l) was used for antagonistic tests and fungal phytopathogens maintaining. Nutrient agar (NA) was used for bacteria culture. Oat kernels were used to prepare an inoculum of the highly pathogenic isolate *P. capsici* (Huang et al., 2001).

Isolation of the endophytic bacterium strain

The healthy plant materials of *H. serrata* (Thunb. ex Murray) Trevis were collected from Lushan Mountain, Jiangxi province, China. Then the plant samples were placed in a plastic bag within an ice box and transported to the laboratory as soon as possible. The samples were thoroughly washed using distilled water and followed by 70% ethanol (v/v) for 2 min and 0.1% mercuric chloride (v/v) for 10 min to accomplish surface sterilization. Plant samples were subsequently rinsed three times in sterile demineralized water. Small pieces of inner tissues were placed on potato dextrose agar and the plates were incubated at 28°C for 2 - 7 days. Samples without surface sterilization were cultured in the same condition as negative controls to check the presence of contaminated microbes on the surface.

After incubation, the endophytic bacterial colony appeared and showed antimicrobial activity against other isolates of endophytic fungi which had been isolated at the same time. The bacterial colony was picked out, streaked on NA and incubated at 28°C for 2 days to get the pure culture. After purification, the bacterial isolate (named as H-6) was cultivated in 5 ml of NA liquid medium with constant shaking at 28°C for 2 days. The culture was suspended in 20% glycerol solution and stored at -80°C.

Antifungal action of strain H-6 *in vitro*

The inoculum of H-6 was prepared from cultures incubated in NA liquid medium at 28°C for 3 - 5 days with constant shaking. 20ml of the fresh liquid culture was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was removed to a new centrifuge tube and filter sterilized. Both the bacterial inoculum and supernatant were used for the dual culture tests.

For the antifungal assay, a plug of mycelium of each fungus (5 mm diameter) was plated at the centre of the Petri dish containing 25 ml PDA and 50 µl liquid endophytic bacterial cultures or supernatant aliquots were put into the holes (5 mm diameter) 3 cm away from the centre. All the tested plates were incubated at 28°C for 5- 7 days and the inhibition effects were evaluated by measuring the diameters of the inhibition zones.

Characterization and identification of the endophytic bacterium strain H-6

According to the experimental design described by Bergey's Manual of determinative bacteriology (Buchanan and Gibbons, 1984), the endophytic bacterium strain H-6 was studied on the morphology, cultural characteristics, physiological and biochemical properties

and Gram stain.

Total bacterial genomic DNA was isolated followed the method described by Frederick et al. (1995). The 16S rDNA was amplified using polymerase chain reaction (PCR) with the universal primers 27f and 1492r (Devereux et al., 1996). The PCR was done in a thermocycler (MJ Research) using a thermal cyclic condition at 94°C (5 min) followed by 35 cycles at 94°C (30 s), 55°C (40 s) and 72°C (90 s) with a final extension temperature at 72°C for 10 min. The PCR products were cleaned using the PCR Cleanup Kit (Tiangen, Biotech Ltd, Beijing, China) and sequenced with ABI 3730 DNA Analyzer (Shanghai Generay Biotechnology Co., Shanghai, China). The sequences were compared using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Alignments between the sequences were performed by using CLUSTAL W software (Thompson et al., 1997) and a phylogenetic tree was made using PAUP version 3.1.1 (Swofford, 1993).

Pot experiments for biocontrol activity against *P. capsici* of pepper

Pot experiments were performed to test the biological control activity of strain H-6 against *P. capsici* in the greenhouse at 22°C under a 12 h photoperiod. Each pot (15 high, 20 cm diameter) was filled with soil mix (Soil : Peat : Perlite = 1:1:1), containing 4 g *P. capsici*-grown oat kernels. Pepper seeds were surface-sterilized in 70% ethanol for 1 min and 1.2% sodium hypochlorite for 5 min, and rinsed 10 times in sterile tap water. Ten surface sterilized pepper seeds (GB16715) were sown and the seeds were covered with a 1 cm layer of soil. Suspensions of three cell densities of H-6 (approximately 10^6 , 10^8 and 10^{10} CFU ml⁻¹) were applied on the soil surface of the pots (30 ml pot⁻¹) by soil drenching immediately after sowing. Pots were randomly distributed in the glasshouse, and the position changed weekly to avoid any positional effects of the experiment.

In the experiments, two controls were included: (a) Inoculated control with *P. capsici*; and (b) non-inoculated control with *P. capsici*; pots contained 4 g sterilized oat-kernel seeds. Each treatment consisted of three replicates. Pots were watered once a week. Plant growth was monitored by recording the lengths and the fresh weights of roots and shoots after 3 weeks. Also the plants were monitored the development of disease symptoms. Disease severity was assessed using a 0-5 scale: 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear on stems; 2, 30-50% of the entire plant diseased; 3, 51-70% of the entire plant diseased; 4, 71-90% of the plant diseased; and 5, plant dead. The disease index (DI) and the control effect (CE) were calculated using the formula described by Lee et al. (2008).

RESULTS

Isolation of endophytic bacteria and *in vitro* antagonistic activity

In this study, nearly 200 endophytic bacteria were isolated based on their cultural characteristics from different part of *H. serrata*. Of the 200 isolates, over 60 isolates with clear antifungal activity were selected from those cultures. Among them, strain H-6 exhibited the highest antifungal activity which was strongly inhibits the growth of many plant pathogenic fungi such as *S. sclerotiorum*, *F. graminearum*, *S. libertiana*, *P. capsici*, *S. fusarium wilt* and *R. solani* (Figure 1).

We also found that the bioactivities of ferment aliquots were weaker than that of the liquid endophytic bacterial

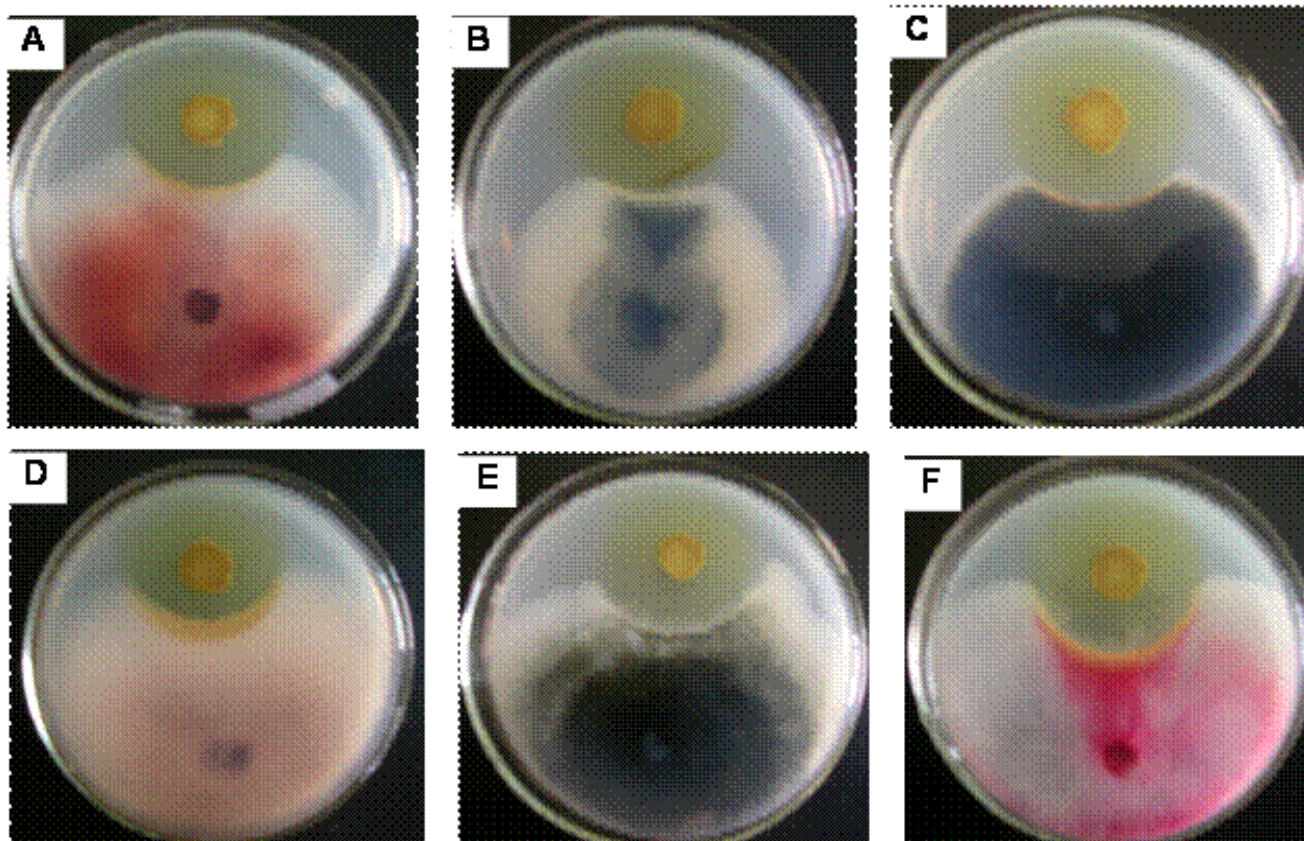


Figure 1. Inhibition effect of H-6 against *Phytophthora capsici* (A), *Rhizoctonia solani* (B), *Sclerotinia scleroliorum* (C), *Sesame fusarium wilt* (D), *Sclerotinia libertiana* (E), and *Fusarium graminearum* (F).

cultures. After analysis of antagonistic activity, *P. capsici* which was intensively inhibited by H-6 was picked out as the model of studying the mechanism of its antimicrobial activity. In the culture dual test plate, the inhibition zones were obvious and the diameters were 23 mm (Figure 1A).

Identification of the endophytic bacterium H-6

The colony of H-6 on LB was yellowish, smooth and opaque. The thallus was rod-shaped and motiled by means of a polar flagellum. In physiological and biochemical studies strain H-6 exhibited for example the following traits: gram negative reaction, catalase positive reaction, the V-P test and nitrate deoxidizing test were also negative, but the M.R. test positive. Strain H-6 was capable of growing on nutrient broth or at temperatures ranging from 10 to 45 °C and fermenting a variety of carbohydrate compounds including glucose, fructose, galactose, mannitol, glycerin, but not maltose and sucrose. Strain H-6 was strictly aerobic, liquefying gelatin, hydrolyzing starch and fiber but not producing H₂S. These properties are typical for *Burkholderia*.

For further identification of H-6, we amplified the 16S rDNA gene sequence and compared the sequence with

sequences from GenBank using BLAST program (Altschul et al., 1990). The 16S rDNA gene sequence of the bacterium strain showed 98.28% identity to that of *Burkholderia* sp. (AJ971348). The resulting phylogenetic tree (Figure 2) showed that H-6 and *Burkholderia* sp. clustered together within one subclade with a bootstrap support of 99%. It was indicated that this strain was phylogenetically related to members of the genus *Burkholderia*.

Control of *P. capsici* by H-6 in greenhouse pot experiments

Soil drenches with the cell densities of H-6 10⁶, 10⁸ and 10¹⁰ CFU ml⁻¹, applied immediately after sowing of pepper seeds, controlled *P. capsici* by 51.7, 58.7 and 60.2%, respectively, compared to the inoculated control treatment. Application of the higher densities of the antagonistic strain proved to be significantly more effective in controlling *P. capsici* compared to the treatment with the lower cell concentration (Table 1). Growth parameters such as lengths and fresh weights of roots and shoots of the *P. capsici*-inoculated control plants were significantly reduced compared to inoculated and treated variants and

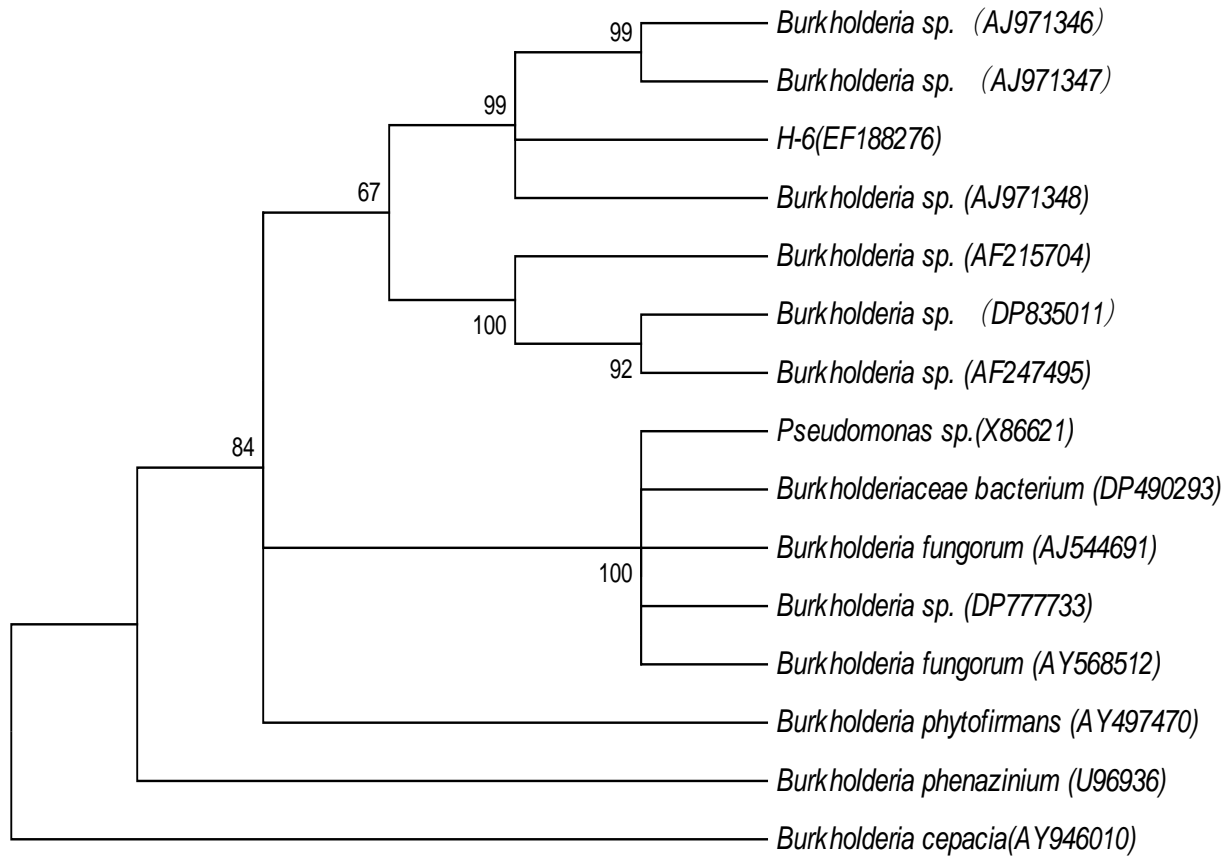


Figure 2. Phylogenetic relationships of the 16S rDNA gene from H-6 (GenBank No. EF188276) and representative related strains from GenBank. *Burkholderia cepacia* (AY946010) was used as outgroup. Percentages above the branches were the frequencies which had been given in 1000 bootstrap replications.

and the latter treatments differed significantly from non inoculated and non-treated control plants (Table 1; Figure 3).

DISCUSSION

Strain H-6, isolated as endophyte from *H. serrata* leaves, has been identified as *Burkholderia* sp. H-6, using morphological, physiological and biochemical methods as well as 16S rDNA analysis. The 16S rDNA gene sequence of the bacterium strain H-6 showed only 91% identity to *Burkholderia cepacia* (AY946010), which might represent a new species and should be more extensively investigated.

Previous studies have shown that strains belonging to the genus *Burkholderia* are effective biological control agents. Such as *B. cepacia*, which is able to antagonize and repress many major soilborne fungal pathogens of crop plants (Hebbar et al., 1992, 1998; Bevivino et al., 1998) and enhance the yield of several crop plants (De Freitas and Germida, 1992; Tabacchioni et al., 1993; Hebbar et al., 1994; Chiarini et al., 1998). Besides, studies also demonstrated that *Burkholderia* spp.

antagonized soil-borne fungal pathogens of crop plants by secreting siderophore (Bevivino et al., 2000; Mendes et al., 2007). Our study revealed that *Burkholderia* sp. H-6 inhibited *in vitro* mycelium growth of numerous plant pathogenic fungi, all the diameter of inhibition zones of the tested pathogens were larger than 15 mm and the inhibition zones of *P. capsici*, *F. graminearum* and *S. libertiana* were up to 23, 22 and 21 mm, respectively. In greenhouse experiments, soil drench treatments with H-6 suspensions (10^6 , 10^8 and 10^{10} CFU ml⁻¹) significantly suppressed *P. capsici* symptoms in pepper between 51.7 and 60.2% compared to the inoculated control. The root growth of soil drench treatments with H-6 suspensions was much better than *P. capsici* inoculated only. Our study preliminary showed that the strain H-6 also can synthesize and secrete siderophore by chromeazuro agar assay method (Schwyn and Neilands, 1987), which was consistent with the former research. Anyway, further work is still required to determine the antagonistic mechanisms of this bacterial strain.

In conclusion, our results obtained from repeated greenhouse pot experiments suggest that strain H-6 could be a candidate biological control agent for the fungal pathogens or may control different plant diseases. How-

Table 1. Effect of soil drench treatments immediately after sowing with the endophytic strain H-6 on seedling growth and *P. capsici* disease of pepper in greenhouse at 22°C.

Treatments	CFU ml ⁻¹	Root		Shoot		DI ± SE	CE (%)
		Lt ± SE (mm)	Fw ± SE (g)	Ht ± SE (mm)	Fw ± SE (g)		
<i>P. capsici</i> inoculated	/	45.5 ± 1.5 A	9.2 ± 0.4 A	64.5 ± 1.2 A	12.4 ± 0.3 A	62.5 ± 1.9 C	0
Non inoculated	/	95.4 ± 1.6 C	23.1 ± 0.6 C	86.3 ± 1.5 D	25.4 ± 0.5 C	0.0	100
<i>P. capsici</i> inoc. + H-6 soil drench	10 ⁶	62.9 ± 1.7 B	13.3 ± 0.5 AB	68.6 ± 1.1 B	19.2 ± 0.2 B	30.2 ± 1.3 B	51.7
<i>P. capsici</i> inoc. + H-6 soil drench	10 ⁸	65.4 ± 1.6 B	16.4 ± 0.8 B	71.4 ± 1.6 BC	23.3 ± 0.4 C	25.8 ± 1.3 A	58.7
<i>P. capsici</i> inoc. + H-6 soil drench	10 ¹⁰	67.7 ± 1.9 B	19.8 ± 0.6 BC	74.1 ± 1.8 C	24.1 ± 0.3 C	24.9 ± 1.5 A	60.2

SE, Standard error of means; CFU, colony forming unit; Lt, length; FW, fresh weight; Ht, height; DI, disease index; CE, control effect (%) compared to inoculated control which was calculated using the formula, CE (%) = 100 x (DI-*P. capsici*-DI-test)/DI-*P. capsici*. Means within a column followed by different letters are significantly different at P = 0.05 according to Fisher's protected least significant difference test.

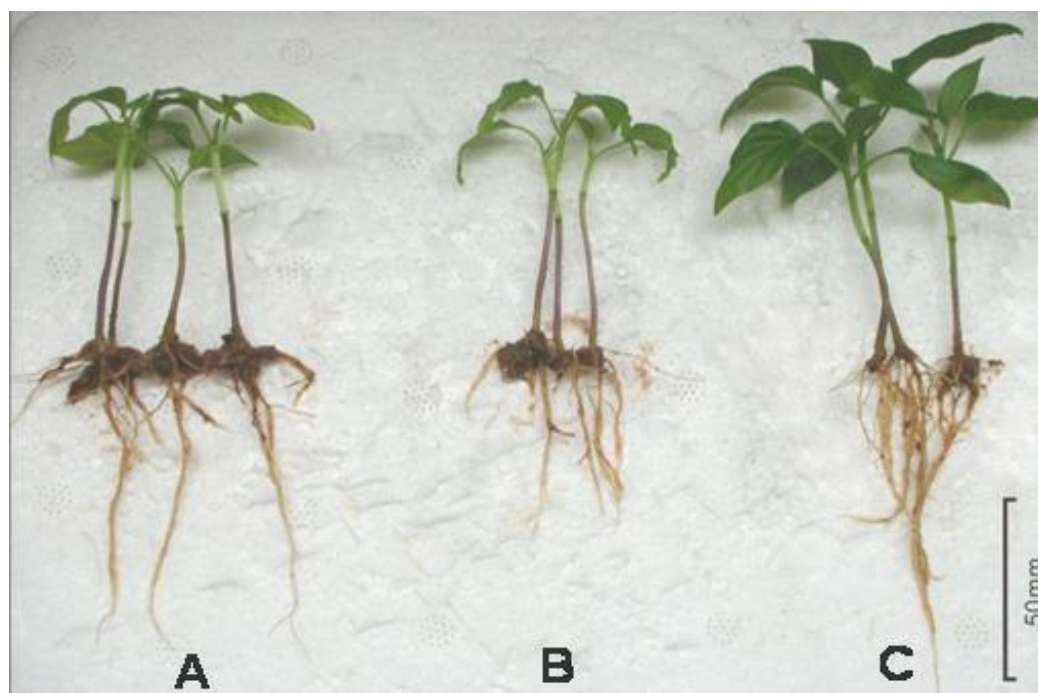


Figure 3. Comparison of growth of pepper seedlings (sampled 21 days after germination) in greenhouse at 22°C inoculated with *P. capsici* and H-6 (10⁸ CFU ml⁻¹) (A). Inoculated with *P. capsici* alone (B); Non-inoculated with *P. capsici* and H-6 (C).

ever, tests based on greenhouse pot experiments do not always correlate with the biological control efficacy under natural conditions. For effective use of strain H-6 inoculant, we need to obtain more knowledge on several fundamental problems ranging from the influence of soil type and other methods of applying inoculum to the understanding of the mechanisms affecting colonization and antagonism in the rhizosphere.

REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
- Assis SMP, Silveira EB, Mariano RLR, Menezes D (1998). Bactérias endofíticas-Método de isolamento e potencial antagonístico no controle da podridão negra em repolho. *Summa. Phytopathol.* 24: 216-220.
- Bagnasco P, De La Fuente L, Gualtieri G, Noya F, Arias A (1998). *Fluorescent Pseudomonas* spp. as biocontrol agents against forage legume root pathogenic fungi. *Soil Biol. Biochem.* 30: 1317-1322.
- Bevivino A, Dalmastrì C, Tabacchioni S, Chiarini L (2000). Efficacy of *Burkholderia cepacia* MCI 7 in disease suppression and growth promotion of maize. *Biol. Fertil. Soils*, 31: 225-231.
- Bevivino A, Sarrocco S, Dalmastrì C, Tabacchioni S, Cantale C, Chiarini L (1998). Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*: effect of seed treatment on disease suppression and growth promotion of maize. *FEMS Microbiol. Ecol.* 27: 225-237.
- Buchanan RE, Gibbons NE (1984). *Bergey's Manual of Determinative Bacteriology*. Beijing Science Publishers, pp. 169-171.
- Chiarini L, Bevivino A, Tabacchioni S, Dalmastrì C (1998). Inoculation of *Burkholderia cepacia*, *Pseudomonas fluorescens* and *Enterobacter* sp. on *Sorghum bicolor*: root colonization and plant growth promotion of dual strain inocula. *Soil Biol. Biochem.* 30: 81-87.
- De Freitas JR, Germida JJ (1992). Growth promotion of winter wheat by *fluorescent pseudomonads* under field conditions. *Soil Biol. Biochem.* 24: 1137-1146.
- Devereux R, Hines ME, Stahl DA, Cycling S (1996). Characterization of Natural Communities of Sulfate-Reducing Bacteria by 16S rRNA Sequence Comparisons. *Microb. Ecol.* 32(3): 283-292.
- Frederick MA, Roger B, Robert EK (1995). *Short Protocols in Molecular Biology*. New York: John Wiley and Sons, pp. 11-13.
- Hallman J, Quadt-Hallmann A, Mahaffee WF, Klopper JW (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* 43: 895-914.
- He RL, Wang GP, Liu XH, Zhang CL, Lin FC (2009). Antagonistic bioactivity of an endophytic bacterium isolated from *Epimedium brevicornu* Maxim. *Afr. J. Biotechnol.* 8(2): 191-195.
- Hebbar KP, Atkinson D, Tucker W, Dart PJ (1992). Suppression of *Fusarium moniliforme* by maize-root associated *Pseudomonas cepacia*. *Soil Biol. Biochem.* 24: 1009-1020.
- Hebbar KP, Martel MH, Heulin T (1994). *Burkholderia cepacia*, a plant growth promoting rhizobacterial associate of maize. In: Ryder MH, Stephens PM, Bowen GD (eds) *Improving plant productivity with rhizosphere bacteria*. CSIRO Division of Soils, Adelaide, Australia, pp. 201-203.
- Hebbar KP, Martel MH, Heulin T (1998). Suppression of pre- and postemergence damping-off in corn by *Burkholderia cepacia*. *Eur. J. Plant Pathol.* 104: 29-36.
- Huang LL, Kang ZS, Heppner C, Buchenauer H (2001). Ultrastructural and immunocytochemical studies on effects of the fungicide Mon65500 (Latitude®) on colonization of roots of wheat seedlings by *Gaeumannomyces graminis* var. *tritici*. *J. Plant Dis. Prot.* 108: 188-203.
- Kavino M, Harish S, Kumar N, Saravanakumar D, Damodaran T, Soorianathasundaram K, Samiyappan R (2007). Rhizosphere and endophytic bacteria for induction of systemic resistance of banana plantlets against bunchy top virus. *Soil Biol. Biochem.* 39: 1087-1098.
- Kong QK, Ding AY (2001). Advances of study on endophytic bacteria as biological control agents. *J. Shandong Agric. Univers Nat. Sci.* 32: 256-260.
- Kudryashava E, Vinokurova N, Ariskina E (2005). *Bacillus subtilis* and phenotypically similar strains producing Hcxacne antibiotics. *Appl. Biochem. Microbiol.* 41: 486-489.
- Lee KJ, Kamala-Kannan S, Sub HS, Seong CK, Lee GW (2008). Biological control of *Phytophthora* blight in red pepper (*Capsicum annuum* L.) using *Bacillus subtilis*. *World J. Microb. Biot.* 24: 1139-1145.
- Mendes R, Pizzirani-Kleiner Aline A, Araujo Wellington L, Raaijmakers Jos M (2007). Diversity of Cultivated Endophytic Bacteria from Sugarcane: Genetic and Biochemical Characterization of *Burkholderia cepacia* Complex Isolates. *Appl. Environ. Microbiol.* pp. 7259-7267.
- Rajkumar M, Lee WH, Lee KJ (2005). Screening of bacterial antagonists for biological control of *Phytophthora* blight of pepper. *J. Basic. Microbiol.* 45: 55-63.
- Rocha R, Luz DE, Engels C, Pileggi SAV, Filho DSJ; Rodrigues MR, Marcos P (2009). Selection of endophytic fungi from comfrey (*Symphytum officinale* L.) for *in vitro* biological control of the phytopathogen *Sclerotinia sclerotiorum* (lib.). *Braz. J. Microbiol.* 40: 73-78.
- Schwyn B, Neilands J (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Chem.* 160: 47-56.
- Strobel G, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* 67: 491-502.
- Sturz AV, Christie BR, Nowak J (2000). Bacterial endophytes: potential role in developing sustainable systems of crop production, *Crit. Rev. Plant Sci.* 19: 1-30.
- Swofford DL (1993). *Phylogenetic analysis using parsimony (PAUP version 3±1.1)*. Champaign, IL, USA: Illinois National History Survey.
- Tabacchioni S, Bevivino A, Chiarini L, Visca P, Del Gallo M (1993). Characteristics of two rhizosphere isolates of *Pseudomonas cepacia* and their potential plant-growth-promoting activity. *Microb. Rel.* 2: 161-168.
- Thomashow LS, Weller DM (1995). Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Stacey G, Keen NT eds. *Plant-microbe interactions*. New York, NY, USA: Chapman and Hall, pp. 187-235.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876-4882.