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# Endophytic bacteria from *Piper tuberculatum* Jacq.: isolation, molecular characterization, and *in vitro* screening for the control of *Fusarium solani* f. sp *piperis*, the causal agent of root rot disease in black pepper (*Piper nigrum* L.)

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**ABSTRACT.** Endophytic bacteria have been found to colonize internal tissues in many different plants, where they can have several beneficial effects, including defense against pathogens. In this study, we aimed to identify endophytic bacteria associated with roots of the tropical piperaceae *Piper tuberculatum*, which is known for its

 

resistance to infection by *Fusarium solani* f. sp *piperis*, the causal agent of black pepper (*Piper nigrum*) root rot disease in the Amazon region. Based on 16S rRNA gene sequence analysis, we isolated endophytes belonging to 13 genera: *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Ralstonia*, *Serratia*, *Cupriavidus*, *Mitsuaria*, *Pantoea*, and *Staphylococcus*. The results showed that 56.52% of isolates were associated with the phylum *Proteobacteria*, which comprised α, β, and γ classes. Other bacteria were related to the phylum *Firmicutes*, including *Bacillus*, which was the most abundant genus among all isolates. Antagonistic assays revealed that Pt12 and Pt13 isolates, identified as *Pseudomonas putida* and *Pseudomonas* sp, respectively, were able to inhibit *F. solani* f. sp *piperis* growth *in vitro*. We describe, for the first time, the molecular identification of 23 endophytic bacteria from *P. tuberculatum,* among which two *Pseudomonas* species have the potential to control the pathogen responsible for root rot disease in black pepper in the Amazon region.

**Key words:** Black pepper**;** 16S rRNA gene; *Fusarium solani* f. sp *piperis*; *Piper tuberculatum*; *Pseudomonas*; Root rot disease

## INTRODUCTION

Endophytic bacteria have been found to colonize the internal tissues of every plant studied. Unlike phytopathogens, endophytic bacteria do not cause visible harm to the host; in contrast, they can exert several beneficial effects on the plant, including defense against pathogens and increased growth and development through the production of plant growth-promoting substances and/or by fixing nitrogen from the atmosphere (Glick, 2012; Mercado-Blanco and Lugtenberg, 2014). Endophytic bacteria can control plant diseases via antagonistic mechanisms, including competition, antibiosis, parasitism, and cross-protection, which result in induced systemic resistance (ISR) (Silo-Suh et al., 1994; van Loon et al., 1998; Verhagen et al., 2010).

The study of endophytes is important to understand their ecological role in nature, and for the identification of new microorganisms that have potential biotechnological applications (Mercado-Blanco and Lugtenberg, 2014). In addition, molecular biology techniques based on polymerase chain reaction (PCR) amplification, including use of the 16S rRNA sequence, have contributed to the precise identification of endophytic bacterial communities (Cho et al., 2007; Kang et al., 2007).

Endophytic bacteria have been isolated from many plant species, such as citrus (Trivedi et al., 2011), coffee (Vega et al., 2005), ginseng (Cho et al., 2007), sugarcane (Hassan et al., 2011), coleus (Vanitha and Ramjegathesh, 2014), and black pepper (Benchimol et al., 2000).

Black pepper (*Piper nigrum* L.) is one of the most widely used spices in the world. The Piperaceae family comprises about 1400 species (Soltis et al., 1999) distributed mainly in the American tropics and Southern Asia, from where black pepper originated (Jaramillo and Manos, 2001). Studies aiming to isolate endophytic bacteria that have the potential to control diseases of black pepper have been reported. For example, Benchimol et al. (2000) identified a *Methylobacterium radiotolerans* bacterium, which reduced mortality of black pepper seed-

lings caused by root rot disease. Antagonistic bacteria for the biological control of nursery wilt in black pepper were isolated by Anith et al. (2003). In addition, Aravind et al. (2009) isolated *Bacillus* and *Pseudomonas* bacteria that are effective in the biological control of *Phytophthora capsici*, the causal agent of the black pepper foot rot disease in India and other Asian countries.

Although significant advances have been made in the identification of endophytic bacteria of black pepper, little is known about endophytes of Piperaceae from the American tropics. Among them, *Piper tuberculatum* Jacq. is known for its resistance to infection by *Fusarium solani* f. sp *piperis* (Albuquerque et al., 2001), which is the causal agent of rot root disease in black pepper, and leads to root rot, leaf fall, and plant death (Albuquerque and Ferraz, 1976).

Pará State, in the Amazon region, is the main Brazilian producer of black pepper; however, its production has been affected by root rot disease (IBGE, 2014). This crop was introduced in Brazil in the 17th century and is currently of significant social and economic importance in the Amazon region, where the pathogen *F. solani* f. sp *piperis* is endemic. Since susceptibility to root rot disease was found in the germplasm collection of all 35 black pepper cultivars available in Brazil (Albuquerque et al., 2001), *Piper* species native to the Amazon region, including *P. tuberculatum*, comprise an important source of resistance to infection, for use in disease control.

Therefore, in this study, we aimed to identify endophytic bacteria of *P. tuberculatum* with the potential to control *F. solani* f. sp *piperis*. Endophytic bacteria isolated from *P. tuberculatum* roots were identified using 16S rRNA gene sequencing, and their ability to inhibit fungal growth was evaluated.

## MATERIAL AND METHODS

### Plant material and isolation of root-associated bacteria

Healthy *P. tuberculatum* plants were harvested from a secondary forest (Ananindeua, Pará, Brazil). Roots were washed in running tap water to eliminate soil residue and other particles. Next, the root surface was disinfected with 70% ethanol (v/v) for 1 min, 2% sodium hypochlorite (v/v) for 6 min, followed by five rinses in sterile distilled water. The efficiency of the disinfection process was checked by plating aliquots of the sterile distilled water used in the final rinse onto nutrient agar (NA) and tryptic soy agar (TSA) media (both supplied by Difco, USA), containing Benomyl (50 mg/mL) to inhibit fungal growth. Plates were incubated at 28°C for 7 days. To eliminate external contamination of roots, their tips were excised using sterile razor blades. Next, disinfected roots were macerated by a pestle in sterile phosphate-buffered saline (PBS 1X: 137 mM NaCl, 10 mM $Na_2HPO4$, 1.8 mM $KH_2$ $PO_4$, 2.7 mM KCl, pH 7.4), followed by incubation at 28°C for 1 h under agitation (150 rpm). Serial dilutions of the bacterial suspension were plated onto both NA and TSA, containing Benomyl (50 mg/mL) and the plates were incubated at 28°C. Bacterial growth was initially assessed after 48 h and extended for until seven days of incubation. Single colonies were isolated and grouped based on growth rate and phenotypic characteristics, such as colony morphology, color, opacity, and size.

### Isolation of DNA from endophytic bacteria

Isolated endophytic bacteria were cultured and collected by centrifugation at 13,000 *g* for 1 min. Cell pellets were resuspended in TE buffer (50 mM NaCl, 10 mM Tris HCl, 50

mM EDTA, pH 8.0), and boiled for 5 min to induce cell lysis. After the addition of RNase and SDS solutions, cell extracts were incubated at 65°C for 30-60 min. The DNA-containing solution was extracted twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol, followed by alcohol-precipitation from the aqueous phase. DNA was collected by centrifugation at 13,000 *g* for 20 min, dissolved in ultra-pure sterile water, and stored at -20°C.

### Amplification of the 16 rRNA gene and sequence analysis

To amplify the 16S rRNA gene, a pair of PCR primers, Y1F (5'-TGGCTCAGAACG AACGCTGGCGGC-3') and Y3R (5'-TACCTTGTTACGACTTCACCCCAGTC-3') (Cruz et al., 2001), was used. The PCR assay conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and final extension at 72°C for 20 min. PCR products were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced using the Big Dye Terminator kit (Applied Systems, USA). Nucleotide sequences were compared to a database using the BLAST program from the NCBI (Altschul et al., 1990). Phylogenetic analysis was performed using the computer software Mega v. 5.04 (Tamura et al., 2011) using the neighbor-joining method. In order to evaluate the stability of nodes, we performed bootstrap analysis with 1000 replicates.

### Inhibition of *F. solani* f. sp *piperis* growth

Endophytic bacteria were tested for their ability to inhibit *F. solani* f. sp *piperis* growth using plates containing potato-dextrose-agar (PDA) medium. A 0.8-cm diameter mycelium disc was placed at the center of a PDA plate and bacterial colonies were streaked 1 cm from the borders of the plate. As a negative control, PDA plates containing mycelium disc with no bacteria were used. There were five repetitions per treatment. Plates were incubated at 28°C for 12 days. Anti-fungal activity was estimated by measuring radial growth inhibition of *F. solani* f. sp *piperis*. To determine differences in radial growth between samples and controls, statistical analyzes were performed using Tukey's test for comparison of means. Relative inhibition (RI) was calculated on the basis of growth in control plates as:

$$RI\ (\%) = \frac{\text{Radial growth in control - Radial growth in samples}}{\text{Radial growth in control}} \times 100$$

## RESULTS AND DISCUSSION

### Isolation of endophytic bacteria associated with *P. tuberculatum* roots

In this study, we aimed to identify endophytic bacteria associated with the roots of *P. tuberculatum* that have potential to control *F. solani* f. sp *piperis*, which causes root rot disease of black pepper in the Amazon region, and leads to severe loss in crop production, and consequently affects the Brazilian economy.

To eliminate contamination by non-endophytic bacteria, the roots were surface-sterilized and the efficiency of the sterilization process was confirmed by the absence of bacterial growth on NA and TSA plates containing aliquots of the sterile distilled water that was used

for the final rinsing of roots. Bacteria colonies grown on NA and TSA plates were first selected based on their morphological characteristics and growth rate. Some bacteria were observed after 48 h of incubation at 28°C, while others exhibited extremely slow growth and were detected only after one week of culture. We isolated 28 morphologically distinct colonies.

Genomic DNA was extracted from these colonies and the 16S rRNA gene was amplified and sequenced. Next, amplified fragments were used to perform a comparative sequence analysis with sequences available in GenBank using the BLASTN program from the NCBI. Our results identified bacteria belonging to *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Ralstonia*, *Serratia*, *Cupriavidus*, *Mitsuaria*, *Pantoea*, and *Staphylococcus* genera. The 16S rRNA gene sequences of 23 *P. tuberculatum* endophytic bacteria were registered in the GenBank databases under the accession No.: JF900600-JF900622.

As shown in Table 1, the identity between the 16S rRNA gene sequences obtained here and those available in GenBank ranged from 94 to 99%. Our results revealed that 23 endophytic bacteria belonging to 13 genera were isolated from *P. tuberculatum* grown in the Amazon region, where a high diversity of plant-associated microorganisms is expected. Many species of endophytic bacteria can be isolated from a single plant, and many factors, such as soil condition, phytopathogen population (Granér et al., 2003), plant age (Islam et al., 2010), host genotype, and plant variety can contribute to significant differences in endophytic bacterial diversity (Adams and Kloepper, 2002). In this context, studies have revealed various numbers of endophytic bacteria species in diverse plants. For example, 63 different endophytic isolates belonging to 13 different bacterial genera were isolated from ginseng roots (Cho et al., 2007); while 87 endophytic bacteria isolates from 19 genera were obtained from coffee plants collected in Colombia and Mexico (Vega et al., 2005). Therefore, the diversity of endophytic bacteria associated with *P. tuberculatum* roots observed in the present study is consistent with that reported in other crops.

**Table 1.** 16S rRNA gene sequence similarities of endophytic bacteria of *Piper tuberculatum* and sequences available in GenBank. Endophytic bacteria were isolated from roots and cultured on TSA and NA plates.

| Isolates | Medium | 16S rRNA gene (bp) | Best match in GenBank Database | Sequence identity (%) |
|---|---|---|---|---|
| Pt1 (JF900600) | TSA | 1398 | *Bacillus* sp strain TZQ22 (HQ143630.1) | 99 |
| Pt2 (JF900601) | TSA | 1411 | *Bacillus* sp strain SuP1 (EU912461.1) | 94 |
| Pt3 (JF900602) | TSA | 1410 | *Bacillus cereus* strain S72 (FJ763650.1) | 99 |
| Pt4 (JF900603) | NA | 1402 | *Bacillus niacini* strain BIHB 356 (FJ859698.2) | 99 |
| Pt5 (JF900604) | NA | 1369 | *Bacillus acidiceler* strain CBD 119 (DQ374637.1) | 98 |
| Pt6 (JF900605) | NA | 1401 | *Bacillus megaterium* strain E5 (JF416939.1) | 99 |
| Pt7 (JF900606) | NA | 1223 | *Bacillus cereus* strain R10 (FR749846.1) | 97 |
| Pt8 (JF900607) | TSA | 1379 | *Paenibacillus* sp strain TDSAS2-39 (GQ284529.1) | 99 |
| Pt9 (JF900608) | TSA | 1391 | *Paenibacillus* sp strain TSWCW18 (GQ284463.1) | 99 |
| Pt10 (JF900609) | NA | 1398 | *Staphylococcus epidermidis* strain BQN1R-01d (FJ380968.1) | 99 |
| Pt11 (JF900610) | TSA | 1362 | *Pseudomonas putida* strain WAB1949 (AM184288.1) | 99 |
| Pt12 (JF900611) | TSA | 1323 | *Pseudomonas putida* strain BJ10 (HQ848377.1) | 99 |
| Pt13 (JF900612) | TSA | 1404 | *Pseudomonas* sp strain TA12-C (HM219617.1) | 99 |
| Pt14 (JF900613) | NA | 1382 | *Pantoea agglomerans* strain SZ009 (EU596536.1) | 98 |
| Pt15 (JF900614) | TSA | 1309 | *Enterobacter* sp strain BBTR105 (EF471230.1) | 99 |
| Pt16 (JF900615) | NA | 1404 | *Serratia marcescens* strain HL1 (EU371058.1) | 99 |
| Pt17 (JF900616) | NA | 1346 | *Rhizobium lusitanum* strain CCBAU 15087 (GU552881.1) | 99 |
| Pt18 (JF900617) | NA | 1330 | *Rhizobium lusitanum* strain BLN7 (GQ181047.1) | 99 |
| Pt19 (JF900618) | NA | 1329 | *Agrobacterium rhizogenes* strain CAF448 (FJ405385.1) | 99 |
| Pt20 (JF900619) | NA | 1338 | *Sinorhizobium* sp strain 2-1 (HM151907.1) | 99 |
| Pt21 (JF900620) | NA | 1319 | *Ralstonia* sp strain SK1 (DQ026295.1) | 99 |
| Pt22 (JF900621) | NA | 1370 | *Mitsuaria chitosanitabida* strain IAM 14711T (AM501442.1) | 99 |
| Pt23 (JF900622) | NA | 1371 | *Cupriavidus pauculus* strain KPS201 (AM418462.1) | 99 |

Among the endophytes identified in the present study, 14 bacteria were isolated on NA plates, and nine were isolated on TSA plates (Table 1). The genus *Bacillus* was isolated from NA and TSA media, while *Pseudomonas, Paenibacillus*, and *Enterobacter* were restricted to TSA medium. Bacteria growing only on NA medium were *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Ralstonia*, *Serratia*, *Cupriavidus*, *Mitsuaria*, *Pantoea*, and *Staphylococcus.* Therefore, the highest number of bacterial genera was found when NA medium was used. Several studies have used different culture media for the successful isolation of endophytic bacteria. For example, Trivedi et al. (2011) reported that NA medium can be used to isolate several citrus root endophytes that had potential to enhance plant growth and suppress disease. Likewise, TSA medium was efficient in isolating many endophytic bacteria from pepper, such as *Pantoea*, *Pseudomonas*, and *Ralstonia* (Kang et al., 2007). In conclusion, although there are data in the literature that demonstrate the efficiency of different media in the isolation of endophytic bacteria, the use of separate plates containing NA or TSA media was advantageous in the present study as it facilitated isolation of diverse species of *P. tuberculatum* endophytic bacteria.

## Endophytic bacteria of *P. tuberculatum* are associated with *Proteobacteria* and *Firmicutes* phyla

A phylogenetic analysis was performed using the 16S rRNA gene sequences obtained in this study and those available in GenBank (Table 1). Our results revealed that endophytic bacteria are associated with the phyla *Proteobacteria* and *Firmicutes* (Figure 1).

Thirteen isolates (56.52% of total) were associated with the phylum *Proteobacteria*, which comprised α, β, and γ classes with four, three, and six bacteria, respectively. Bacteria related to the *γ-Proteobacteria* class included *Pseudomonas putida* (Pt11 and Pt12), *Pseudomonas* sp (Pt13), *Pantoea agglomerans* (Pt14), *Enterobacter* sp (Pt15), and *Serratia marcescens* (Pt16). *Pseudomonas* is a well-known plant-associated bacterium found in the majority of plant species. These bacteria have been effective in the biological control of diverse diseases, such as foot rot in black pepper (Aravind et al., 2009) and grapevine gray mold caused by *Botrytis cinerea* (Verhagen et al., 2010). Additionally, *Pseudomonas* bacteria are considered plant growth-promoting bacteria (PGPB) due to many traits, which contribute to the growth and development of plants, such as the production of phytohormones and siderophores (Glick, 2012). *Serratia marcescens* and *Pantoea agglomerans* are also PGPB, since they can control plant disease (Hsieh et al., 2005) and promote plant growth (Selvakumar et al., 2007), cause solubilization of phosphates (Tripura et al., 2007), and cold tolerance (Selvakumar et al., 2007). We also identified four potential PGPB (Pt17, 18, 19, and 20) that are associated with the *α-Proteobacteria* class. These four isolates were associated with the bacteria *Rhizobium lusitanum, Agrobacterium rhizogenes,* and *Sinorhizobium* sp, which are known to contribute to plant growth and development by fixing atmospheric $N_2$ (Kanvinde and Sastry, 1990; Valverde et al., 2006; Talebi et al., 2008). Endophytic bacteria related to the *β-proteobacteria* class included the Pt21, 22, and 23 isolates. The bacterium *Ralstonia* has been found as PGPB in many crops, such as pepper (Kang et al., 2007). Hence, based on the literature, we can conclude that most isolated *Proteobacteria* that are associated with *P. tuberculatum* have the potential to contribute to defense against pathogens and/or the promotion of plant growth and development.

Endophytic bacteria affiliated with the phylum *Firmicutes* comprised 43.48% of the total isolates, and included the genera *Bacillus*, *Paenibacillus,* and *Staphylococcus*. *Bacillus* was the most abundant genus found and comprised seven isolates: *B*. sp (Pt1 and Pt2), *B. ce-*

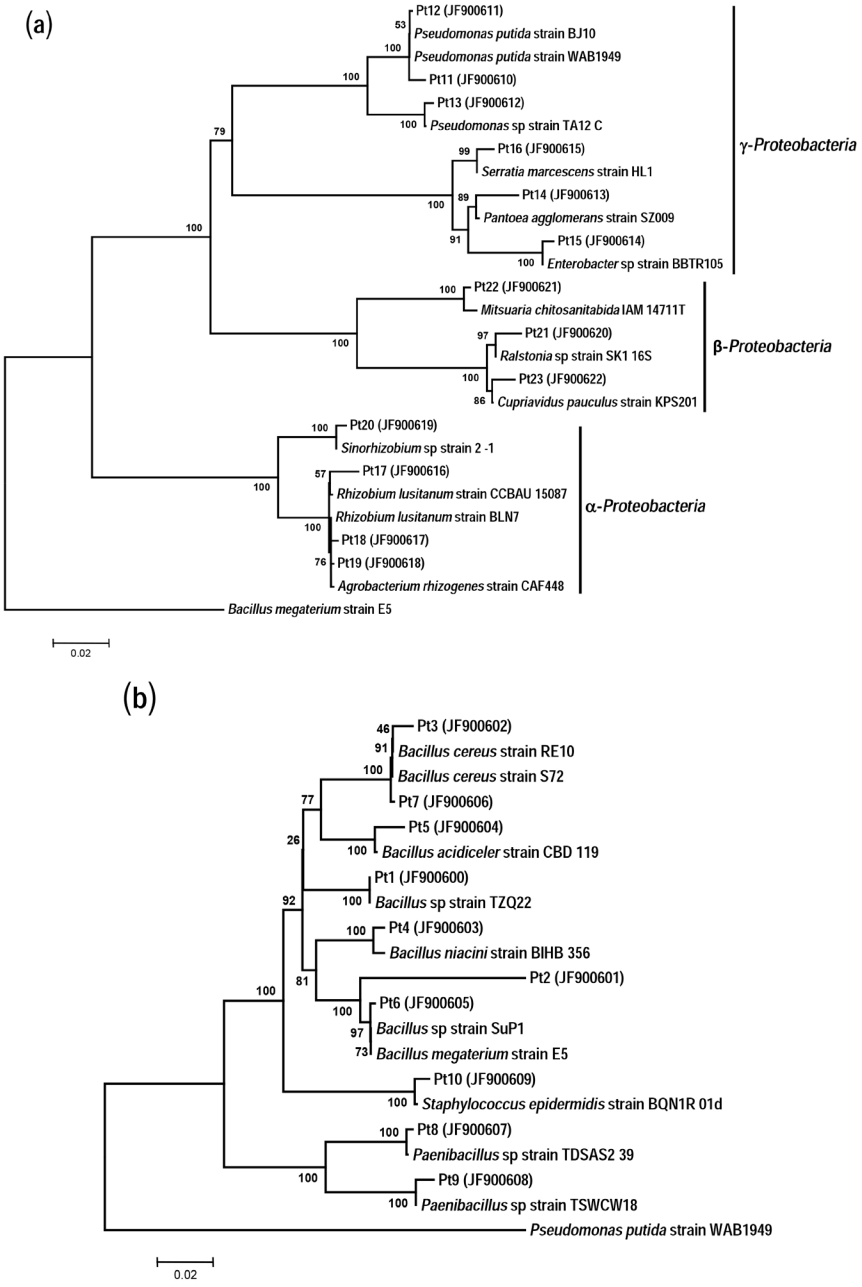


**Figure 1.** 16S rDNA-based dendrogram showing the phylogenetic relationship of endophytic bacteria from *Piper tuberculatum* roots and sequences available in GenBank Database cited in Table 1. Phylogenetic analysis was performed using the software Mega v. 5.04 and neighbor-joining method. The scale bar represents a 2% estimated difference in nucleotide sequence. Numbers above each node indicate bootstrap values. Sequences of *Bacillus megaterium* strain E5 (JF416939.1) and *Pseudomonas putida* strain WAB1949 (AM184288.1) were used as outgroup references for *Proteobacteria* (**a**) and *Firmicutes* (**b**) phyla, respectively.

*reus* (Pt3 and Pt7), *B. niacini* (Pt4), *B. acidiceler* (Pt5), and *B. megaterium* (Pt6). The Pt8 and Pt9 isolates matched those of *Paenibacillus*. *Paenibacillus* and *Bacillus* are known to protect plants against phytopathogenic fungi using different mechanisms, including chitinolytic activity (Huang et al., 2005; Singh et al., 2009) and via the production of fungistatic antibiotics (Silo-Suh et al., 1994). Therefore, among endophytic bacteria that were associated with the phylum *Firmicutes* isolated in this study, *Bacillus* and *Paenibacillus* are candidates that might be involved in the protection of *P. tuberculatum* against pathogens.

## *Pseudomonas* bacteria can inhibit *in vitro* growth of *F. solani* f. sp *piperis*

We performed antagonistic assays to screen *P. tuberculatum* endophytic bacteria for the ability to inhibit *F. solani* f. sp *piperis* growth. For these assays, we used Pt1 to Pt16 isolates, except Pt10, identified as *S. epidermidis*, which is part of the normal human flora (Fey and Olson, 2010). These 15 isolates were selected for use in antagonistic assays because they shared the 16S rRNA sequence identity with bacteria that have known roles in the biological control of phytopathogens.

Our results revealed that Pt12 and Pt13 isolates, identified as *P. putida and P.* sp, respectively, were able to inhibit radial growth of *F. solani* f. sp *piperis* on PDA medium, when compared to the negative control, i.e., without endophytic bacteria (Figure 2). A similar result was obtained when we compared these two isolates with the Pt3 isolate, identified as *B. cereus*, which showed no inhibition of fungal growth (Figure 2). Statistical analysis using Tukey's test revealed inhibition of fungal growth at 38.96% and 55.31% by Pt12 and Pt13 isolates, respectively (Table 2). These results are in accordance with those obtained in other studies, including a recent report by Vanitha and Ramjegathesh (2014), in which the inhibitory activity of *Pseudomonas* strains isolated from coleus rhizosphere against the fungus *Macrophomina phaseolina* ranged from 30 to 60%. In addition, studies have reported the effective use of *Pseudomonas* bacteria, including *P. putida* strains, in the biological control of pathogens of several plants, such as cotton (Chen et al., 1995), black pepper (Tran et al., 2008), sugarcane (Hassan et al., 2011), and coleus (Vanitha and Ramjegathesh, 2014).

Although it was not the goal of the present study to elucidate the mechanisms by which Pt12 and Pt13 isolates were able to inhibit fungal growth, based on the literature, some hypotheses can be considered. For example, studies have shown that the mechanisms of *Pseudomonas* bacterial action in the control of phytopathogens include production of biosurfactant compounds (Tran et al., 2008) and the antibiotic pyoluteorin (Hassan et al., 2011). Another mechanism by which *Pseudomonas* bacteria can protect plants against pathogens is via the ISR (Kang et al., 2007; Verhagen et al., 2010), where plants inoculated with endophytic bacteria, followed by contact with pathogens, showed enhanced expression of genes involved in the ISR, indicating that these genes were primed to respond more intensely to pathogen attack. Changes frequently observed in plants during the ISR include structural changes in cell walls, enhanced activity of enzymes related to defense, and enhanced phytoalexin accumulation (van Loon et al., 1998; Liu et al., 2007; Verhagen et al., 2010). Interestingly, a recent study by Schenk et al. (2014) showed that *N*-acyl-homoserine lactone, a bacterial quorum-sensing molecule, was able to prime cell wall-related genes in *Arabidopsis thaliana* plants and, as a result, induce resistance to bacterial pathogens by way of cell wall reinforcement. Since we have identified sequences of *P. tuberculatum* that code for proteins potentially involved in the formation of structural barriers against *F. solani* f. sp *piperis* infection, including a putative

cell wall hydroxyproline-rich glycoprotein (Nascimento et al., 2009); it is possible that the *Pseudomonas* bacteria isolated in the present study contribute to resistance to this fungus by similar mechanisms. However, further studies will elucidate the roles of Pt12 and Pt13 isolates in the physiology of *P. tuberculatum*.

To the best of our knowledge, this study is the first report on the molecular identification of 23 endophytic bacteria from tropical *P. tuberculatum,* among which, two *Pseudomonas* bacteria were isolated that have the potential to control the pathogen responsible for root rot disease of black pepper in the Amazon region.

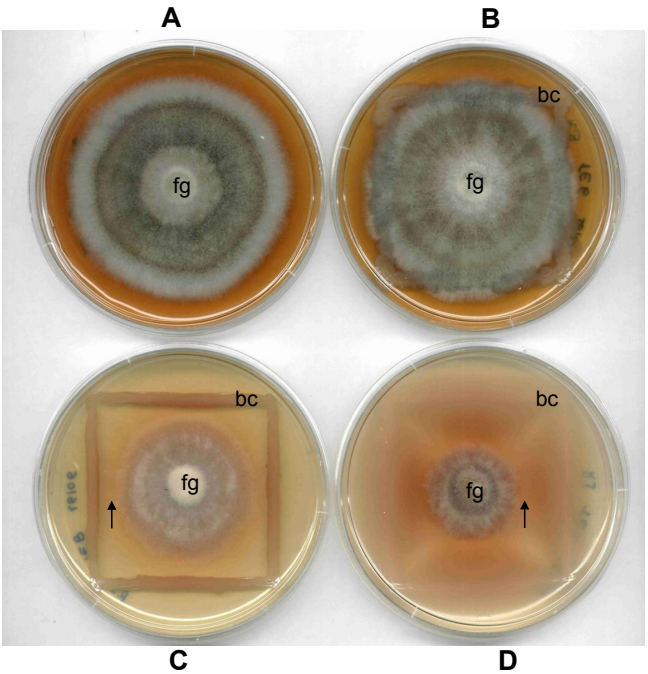


**Figure 2.** Assays of inhibition of *Fusarium solani* f. sp *piperis* growth by endophytic bacteria associated with roots of *Piper tuberculatum* on PDA medium at 12 days after incubation. **A.** *F. solani* f. sp *piperis* (control), **B.** *F. solani* f. sp *piperis* + Pt3 isolate (*B. cereus*), **C.** *F. solani* f. sp *piperis* + Pt12 isolate (*P. putida*) and **D.** *F. solani* f. sp *piperis* + Pt13 isolate (*P.* sp). bc: bacterium, fg: fungus. The arrows point to the free zones.

**Table 2.** Inhibition of *Fusarium solani* f. sp *piperis* growth by endophytic bacteria associated with roots of *Piper tuberculatum*.

| Treatments | Radial growth (cm)* | Relative inhibition-RI (%) |
|---|---|---|
| *F. solani* f. sp *piperis* (control) | 7.34[a] | 0.00 |
| *F. solani* f. sp *piperis* + Pt3 isolate (*B. cereus*) | 7.32[a] | 0.27 |
| *F. solani* f. sp *piperis* + Pt12 isolate (*P. putida*) | 4.48[b] | 38.96 |
| *F. solani* f. sp *piperis* + Pt13 isolate (*P.* sp) | 3.28[c] | 55.31 |

*Means followed by different letters within one column are significantly different by Tukey's test at a probability level of 1%. Relative inhibition (RI) was calculated by the following formula: RI (%) = [(Radial growth in control - Radial growth in samples)/Radial growth in control] x 100.

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