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Full Length Article

Vanilla Rhizobacteria as Antagonists against *Fusarium oxysporum* f. sp. *vanillae*

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Abstract

Pathogens such as *Fusarium oxysporum* strongly affect the health of various agricultural crops like vanilla. However, despite significant economic losses caused by this pathogen there is no efficient method for its control. Therefore, we propose using rhizobacteria obtained from vanilla roots against *F. oxysporum* f. sp. *vanillae*. The results showed that there was no positive correlation between the antagonism expressed under *in vitro* conditions and those expressed under greenhouse conditions. The 16S rDNA gene analysis indicated that the bacterial genera tested corresponded to *Sphingobacterium*, *Staphylococcus*, *Serratia*, *Psychrobacter*, *Pseudomonas* and *Stenotrophomonas*. The *in vitro* antifungal activity was evaluated using three culture media (Potato Dextrose Agar, Nutrient Agar and Czapek) using the empty box technique (antagonism). Isolates of *Staphylococcus xylosus* BAC-JAG15, *Serratia* sp. BAC-JAG4 and *Stenotrophomonas* sp. BAC-JAG1 showed 90% *in vitro* antagonism against *F. oxysporum* in the three media tested. In the greenhouse evaluation, plants treated with these isolates initially showed symptoms of chlorosis without developing characteristic symptoms of disease produced by *F. oxysporum* f. sp. *vanillae*. This demonstrates protection provided by rhizobacteria against infection from *F. oxysporum* f. sp. *vanillae* in vanilla plants.

Keywords: Biological control; Soil borne fungal pathogens; Antifungal activity; Vanilla

Introduction

Vanilla (*Vanilla planifolia* Jacks. ex. Andrew) has low genetic variability (Minoo *et al.*, 2008) due to the clonal origin of crops and vegetative propagation, making it susceptible to a large number of diseases caused by *Phytophthora* sp., *Calospora vanillae* Massee, *Sclerotium* spp., *Colletotrichum gloeosporioides* (Penz.) Sacc. and *Fusarium* spp. (Bhai and Thomas, 2000; Summerell *et al.*, 2003; Guzman, 2004; Talubnak and Soytong, 2010).

Fusarium oxysporum is the most important pathogen responsible for severe damage to the cultivation of vanilla, and has thus been the most studied genus. However, despite significant economic losses caused by the disease in vanilla, there has not, to date, been an efficient method for controlling this disease. Therefore, it is imperative to investigate the association of rhizosphere manipulation, which contains beneficial microorganisms, with its antagonistic properties that protect roots from harmful effects caused by soil pathogens (Weller, 1988).

The potential uses of plant-associated bacteria as biocontrol agents of plant diseases have been described (Rovira, 1965; Hallmann *et al.*, 1997; Sturz and Nowak, 2000; Welbaum *et al.*, 2004), of which the growth promoting rhizobacteria or PGPR are the most studied (Kloepper and Schroth, 1978). The PGPR colonize root surfaces and/or are closely adhered to the interface between the soil and roots, the rhizosphere (Kloepper and Schroth, 1978; Kloepper *et al.*, 1999).

Bacteria benefit the plant by directly interacting with beneficial microorganisms and the host plant, and through indirect mechanisms such as antifungal activity (Berg, 2009). They can reduce the incidence or severity of plant disease by acting as biological control agents or by exhibiting antagonistic activity towards a pathogen (antagonists) (Beattie, 2006).

Bacteria provide effective protection to plants by employing multiple modes of action such as production of antimicrobial compounds, production of enzymes that interfere with fungal pathogenesis, competition for resources, induction of plant resistance and interactions among members of the microbial community (Beattie, 2006).

Although numerous studies have been focused on using biocontrol against *Fusarium* spp. for various crops, there have been few studies to control this pathogen using rhizobacteria in vanilla. Among studies on the biological control of *F. oxysporum* f. sp. *vanillae* (*Fov*) with different

strains of bacterial species are those by Tombe *et al.* (1997) who determined the effectiveness of *Pseudomonas fluorescens* as a *Fov* antagonist. The results obtained by Bhai and Kumar (2008) showed that *P. fluorescens* was a more effective antagonist to *Phytophthora meadii*, *Fov* and *Colletrotrichum vanillae* than other bacteria such as *Enterobacter agglomerans*, and *Bacillus* spp. However, these studies do not indicate whether the strains used corresponded to microorganisms isolated from root cultures of vanilla or were strains tested on other crops. As an alternative, the use of native species has not been reported for the control of phytopathogenic fungi.

Based on the above, the hypothesis of the present study is that microbial populations in the rhizosphere of vanilla contain bacteria with antagonistic activities that can be used as biocontrol agents against *F. oxysporum* f. sp. *vanillae*. The main objective was to evaluate the antagonistic capacity of rhizobacteria associated with *Vanilla planifolia* Jacks. ex Andrew against *F. oxysporum* f. sp. *vanillae* under *in vitro* conditions and in soil inoculated with the pathogen.

Materials and Methods

Experimental Details and Treatments

Experimental material: The *F. oxysporum* strain used in this work corresponds to *F. oxysporum* f. sp. *vanillae*, which was isolated and reported to be highly pathogenic to vanilla (Adame-García *et al.*, 2011).

Collection and Isolation of Rhizobacteria

Bacterial isolates were obtained from vanilla plant roots from Papantla, Veracruz (20° 21' 47.57" N, 97° 30' 39.06" W), Mexico, a region having high production of this crop. Samples were placed in plastic bags, labeled and transported to the laboratory. Roots were cut into 5 mm fragments and 10 g was weighed out from each root. Each sample was placed into a sterile 250 mL Erlenmeyer flask to which was added 90 mL sterile phosphate solution (0.25 M KH_2PO_4). The solution was stirred at 120 rpm for 10 min in an orbital shaker (Lab Line). Then five dilutions were prepared (1:9), and 0.1 mL of the 10^{-5} and 10^{-6} dilutions were spread onto agar crab shell medium (3, 6 and 9%), which were incubated at 26 ± 1°C for 72 h.

Evaluation of Antifungal Properties

***In vitro* evaluation:** For this evaluation the fungal *vs.* bacterial antagonism produced when grown in culture medium was used.

Antagonism through Competition, Fungi *vs*. Bacteria in Culture Medium

For this determination, the empty box method was used with three media: nutrient agar (NA), potato dextrose agar (PDA) and Czapek Dox. A completely randomized design with five replicates was used for which 100 µL of bacterial solution (1×10^8 UFC mL^{-1}) was used and distributed uniformly in 20 mL for each of the three culture media studied. Once the agar solidified, a sample of mycelium was applied with a dissecting needle. All boxes (treatments and control with no bacteria) were incubated in an incubator (Felisa) at 25±1ºC. The radial mycelial growth was measured at 7 days post-inoculation. Data from *in vitro* tests were analyzed with StatView 5.0 using ANOVA and Tukey tests to determine differences among treatments.

Greenhouse Evaluations

Greenhouse evaluations were conducted in Úrsulo Galván, Veracruz, México, located 8 masl at 19° 24' N and 96° 18' W. The climate in this region is hot sub-humid, with an average annual temperature of 25.8°C and an average annual rainfall of 1,017.7 mm.

A randomized block design was used with 5 replicates (cuttings) and 50 treatments (Fig. 1). All treatments were conducted in black plastic bags (10 × 15 cm) containing 500 g of soil and infected with 10 mL of an *Fov* spore suspension (10^6 spores mL^{-1}) from cultures 12 days old cultured on PDA medium and incubated at 26±1ºC. To obtain the spore suspension, 2 mL of Tween 20 solution (20%) was added to each petri dish containing the mycelia. This solution was then decanted into a test tube containing 8 mL of sterile distilled water and then mixed with a vortex (MAXI-MIX II). From this solution, aqueous suspensions containing 10^6 spores mL^{-1} were prepared. A sterile solution of Tween 20 (20%) without fungal inoculum was used as a control.

Three types of bacterial inoculation were used: (1) cuttings immersed in bacterial suspension for 10 min, (2) cuttings immersed in bacterial suspension for 10 min and re-inoculated three days after planting, and (3) inoculation at the three days after planting. As controls, cuttings inoculated with bacterial isolates from soil not inoculated with *Fov*, and cuttings without bacterial or fungal inoculum were used. Bacterial solutions of 1×10^8 UFC mL^{-1} were used from cultures maintained for 24 h in NA medium.

The test was conducted two days after inoculation of the mycelia in the soil and at different times before planting. With a sterile razor, an incision of 1 cm in length was made in the roots and this portion of the cuttings was placed in contact with the soil. The cuttings were watered three times a week. Temperatures ranged from 26–28°C during the day and 22–24°C at night.

The damage level of the cuttings was evaluated at 60 days after planting, and four levels of damage were identified: (1) cuttings without symptoms, (2) cuttings with symptoms of chlorosis, (3) cuttings with rot, and (4) necrotic or dead cuttings. After the evaluation, Koch's postulates were applied to confirm the presence of the inoculated fungus.

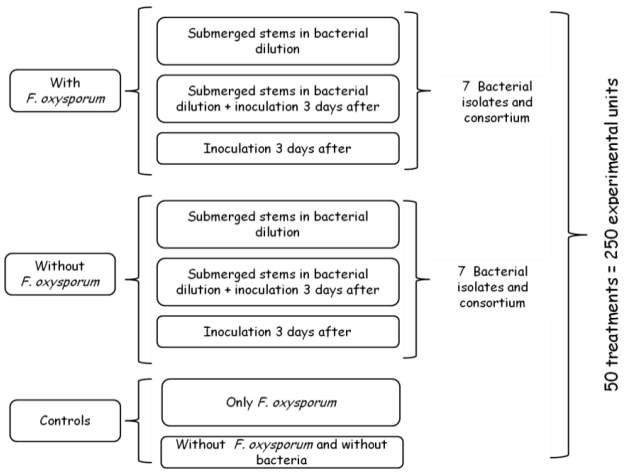

Fig. 1: Treatments established for the evaluation of antifungal capacity in greenhouse conditions

The data were analyzed using Statistica 7.0. The nonparametric Friedman ANOVA and Kendall's Coefficient of Concordance were applied. For nonparametric sample contrasts, the Wilcoxon test was used.

Molecular Identification of Bacterial Isolates

For the molecular identification of isolates, DNA extraction was performed according to Cheng and Jiang (2006). DNA integrity was assessed using 0.8% agarose gel electrophoresis (TBE 0.5 X) in a horizontal chamber (CONSORT) at 100 V. The gels were stained in 100 mL of 1X TBE solution with 2 µL of ethidium bromide (10 mg mL^{-1}) for 20 min, and then observed with a MicroBis photodocumentation system.

Bacterial isolates were selected for their antifungal properties and were applied by Luria plate streaking on Luria Bertani medium (LB). The plates were incubated at 27±1°C in a bacteriological incubator (Felisa).

The amplification of the 16S rDNA gene was performed following Luna *et al.* (2013) and using 0.20 mM of dNTPs and similar thermal cycling conditions. The PCR products were purified using a ChargeSwitch® - Pro PCR Clean-up Kit (Invitrogen) following the manufacturer's instructions and then sequenced at the Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM) using an Applied Biosystems Sequencer (model 391) and employing the BMB-CR oligonucleotide.

The sequence data were edited using BioEdit version 7.0.5.3 (Hall, 1999) and analyzed via the Basic Local Alignment Search Tool (BLAST) system (GenBank, National Center for Biotechnology Information (NCBI).

Phylogenetic and molecular analyses were conducted using MEGA version 5 (Tamura *et al.*, 2011) with the Maximum Parsimony (MP) method. The MP tree was obtained using the Maximum Likelihood method was based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree was inferred from 1,000 replicates (Felsenstein, 1985).

Results

Isolation of Rhizobacterial Strains

Bacterial colonies were visible at 4 days after planting. A total of 116 bacterial isolates were obtained for evaluation of antifungal activity against *Fov*.

***In Vitro* Evaluation, Fungal Antagonism *vs*. Bacterial Culture in Medium**

Only seven of the 116 bacterial isolates tested exhibited inhibition relative to the controls.

Analysis of the 16S rDNA gene identified these isolates as *Sphingobacterium* sp. BAC-JAG26 and BAC-JAG89, *Staphylococcus xylosus* BAC-JAG15, *Serratia* sp. BAC-JAG4, *Psychrobacter* sp. BAC-JAG39, *Pseudomonas* sp. BAC-JAG101, and *Stenotrophomonas* sp. BAC-JAG1.

Evaluation of the antifungal capacity of these strains in the three cultivation media (Fig. 2) revealed significant differences among the isolates (df=7, P<0.0001). In PDA medium, strains of *Serratia* sp. BAC-JAG4 and *Sphingobacterium* sp. BAC-JAG26 reduced growth of *Fov* by 69% and 52%, respectively (Fig. 3a). However, in NA medium, antagonistic action increased slightly for *Serratia* sp. BAC-JAG4 to 75% and markedly for *Sphingobacterium* sp. BAC-JAG26 to 80% (Fig. 3b). Particularly interesting was *S. xylosus* BAC-JAG15 on PDA medium which induced an inhibition of only 32%, contrasting with 94% and 81% in NA and Czapek media, respectively (Figs. 3b, c).

In Czapek medium, some bacterial isolates showed a different trend compared to PDA and NA media. Particularly, *Pseudomonas* sp. BAC-JAG101, *Sphingobacterium* spp. BAC-BAC-JAG26 and BAC-JAG89, which inhibited *Fov* growth, showed no inhibition in Czapek medium (Fig. 3c). *Psychrobacter* sp. BAC-JAG39 had the lowest inhibition percentage (Fig. 3b) on PDA and Czapek media and had a slight stimulatory effect (3%) on fungal growth in NA medium.

Isolates *S. xylosus* BAC-JAG15, *Serratia* sp. BAC-JAG4 and *Stenotrophomonas* sp. BAC-JAG1 reduced the growth of the fungus on PDA and NA media by 94%, and also exerted an inhibitory effect in Czapek medium, by 81%, 77% and 79%, respectively (Fig. 3c).

Greenhouse Evaluation

The results of greenhouse evaluation (Fig. 4) revealed significant differences (df=9, p=0.00821) in the inhibition of *Fov* activity among bacterial isolates, when vanilla cuttings were immersed for 10 min in a bacterial suspension and then planted in soil inoculated with the fungus (Fig. 5a).

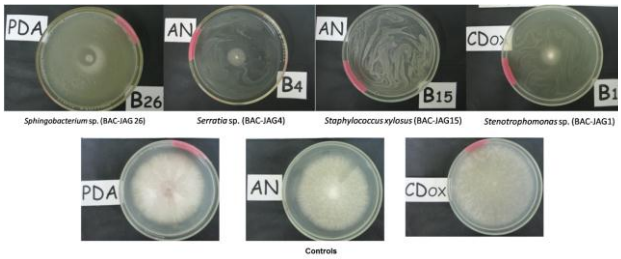

Fig. 2: Appearance of *Fov* strains grown simultaneously with the bacterial isolates that showed the highest percentages of inhibition. Also shown are *Fov* strains without bacterial inoculants (controls)

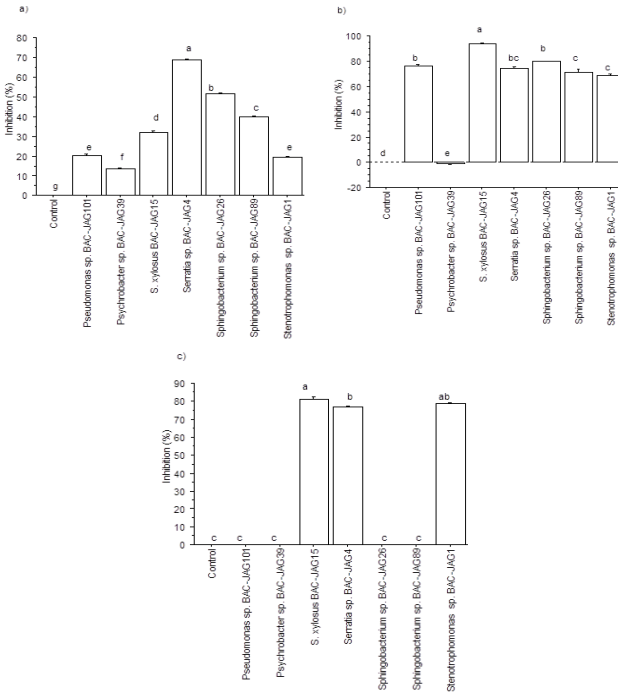

Fig. 3: Inhibition of cultured *Fov* growth simultaneous with the antagonistic bacterial isolates in the three tested media; (a) PDA, (b) NA and (c) Czapek. Bars represent the standard error of five replicates per sample. Different letters indicate significant differences from a Tukey test ($p<0.05$)

Psychrobacter sp. BAC-JAG39 expressed the greatest antifungal properties as there were no significant differences between it and the negative control treatment (Fig. 5a). However, when using other methods of inoculation (three days after planting), the treated cuttings showed signs of chlorosis. This result was also observed in treatments inoculated with *Serratia* sp. BAC-JAG4 (Fig. 5c). Treatments with no favorable results were those where the cuttings were inoculated with *Pseudomonas* sp. using any of the three methods of inoculation, with the cuttings presenting symptoms of chlorosis, including tissue rotting and death (Fig. 5a, b and c).

Fig. 4: (a) Cuttings without symptoms, *Psychrobacter* sp. BAC-JAG39 expressed the greatest antifungal properties; (b) Using any of the three methods of *Pseudomonas* sp. inoculation, cuttings presenting symptoms of rotting and death

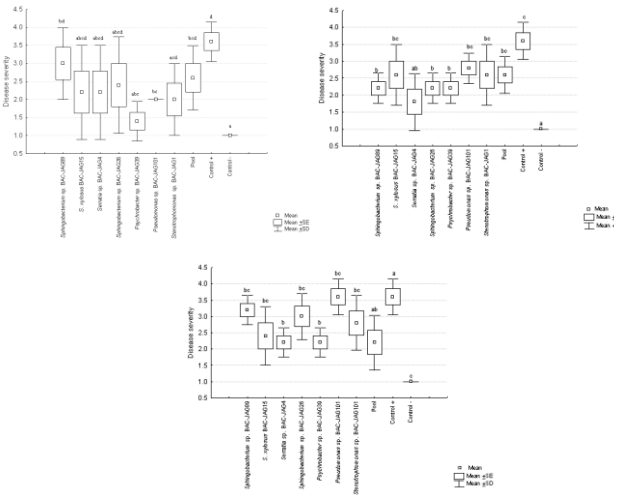

Fig. 5: Levels of vanilla stem damage caused by *Fov* before the presence of bacterial isolates in the three types of inoculation: a) stems immersed in bacterial suspension for 10 min; b) stems immersed in bacterial suspension for 10 minutes and inoculated three days after planting; c) stems inoculated three days after planting. Error bars represent the standard error of five replicates per sample. Different letters indicate significant differences (Wilcoxon test, $p<0.05$)

In treatments where the cuttings were inoculated with the different bacterial suspensions and planted in soil without *Fov* inoculation, significant differences were detected among them (df=9, p=0.0009) that were attributable to a pathogenic from bacterial strain. Such is the case when isolating *Pseudomonas* sp. where the cuttings, without the presence of *Fov*, developed chlorosis (Fig. 6a) and then symptoms of tissue rot (Fig. 6b).

In treatments where the cuttings were immersed in bacterial suspensions of *Sphingobacterium* spp., *S. xylosus* BAC-JAG15 and *Serratia* sp. BAC-JAG4, there were slight symptoms of chlorosis, but these treatments were not significantly different compared to the negative control (Fig. 6a and b). Furthermore, in treatments where the cuttings

were inoculated three days after planting, these bacteria did not produce any damage to vanilla stems (Fig. 6c).

Discussion

In the present study, we used culture media with added crab shell as an alternative to selecting bacterial strains capable of producing extracellular enzymes to hydrolyze the chitin present in the cell walls of fungi (Chang *et al.*, 2003; Wang *et al.*, 2006; Chang *et al.*, 2009; Wang *et al*., 2009; Wang *et al*., 2010) where certain strains of *Bacillus cereus* and *B. subtilis*, when grown in media with crab and/or shrimp shells, produce chitinolytic enzymes efficient for the *in vitro* inhibition of *F. oxysporum*, *F. solani* and *Pythium ultimum*.

Obtaining strains of *Sphingobacterium*, *Psychrobacter*, *Pseudomonas*, *Serratia*, *Staphylococcus* and *Stenotrophomonas* from other plant species using culture media with crab and/or shrimp shells as the only carbon source has been reported (Wolf, 2002; Chang *et al.*, 2003; Ribbeck-Busch *et al.*, 2005; Wang *et al.*, 2006; Chang *et al.*, 2009; Wang *et al.*, 2009; Zachow *et al.*, 2009). Likewise, strains of these genera have been reported as rhizobacteria with *in vitro* antagonistic activity against phytopathogenic fungi, including *F. oxysporum*, but not for the strain *vanillae*. To date, only the strains of *Pseudomonas* sp. have been used as antagonists of *Fov* (Tombe *et al.*, 1997; Bhai and Kumar, 2008).

Here, the results from *Serratia* sp. BAC-JAG4, which reduced fungal growth in the three culture media tested and did not yield symptoms of wilt or rot, are consistent with previous studies reporting that *S. liquefaciens*, *S. plymuthica* and *S. rubidaea* showed *in vitro* antifungal activity against different fungal pathogens (Kalbe *et al.*, 1996). In particular, isolates of *S. plymuthica* have been used as biocontrol agents against *Verticillium dahliae*, *Rhizoctonia solani* and *F. oxysporum* in wheat, oats, cucumber, corn, canola and potato (Åström and Gerhardson, 1988; Kloepper *et al.*, 1992; Kalbe *et al.*, 1996; Berg, 2000; Frankowski *et al.*, 2001; Ting *et al.*, 2011).

The antifungal mode of action for *Serratia* is based on antibiosis (prodigiosin and pyrrolnitrin production) and production of degradative enzymes of fungal cell walls (chitinases and β-1, 3 glucanase). As well, they produce potent siderophores to improve iron availability. However, it has been shown that the mode of action is specific to each species of *Serratia* (Kalbe *et al.*, 1996; Guevara-Avendaño *et al.*, 2014). For example, *S. liquefaciens* and *S. marcescens* produce chitinases similar to those of *S. plymuthica* which has been used to inhibit the germination of spores of the phytopathogenic fungus *Botrytis cinerea* (Frankowski *et al.*, 2001).

Another bacterial isolate that provided antagonism in the three media (PDA 32%, NA 94%, Czapek 81%) was *S. xylosus* BAC-JAG15. However, this species is commonly found in foods, as it is one of the main agents used for the fermentation of meat; it has not yet been reported as a

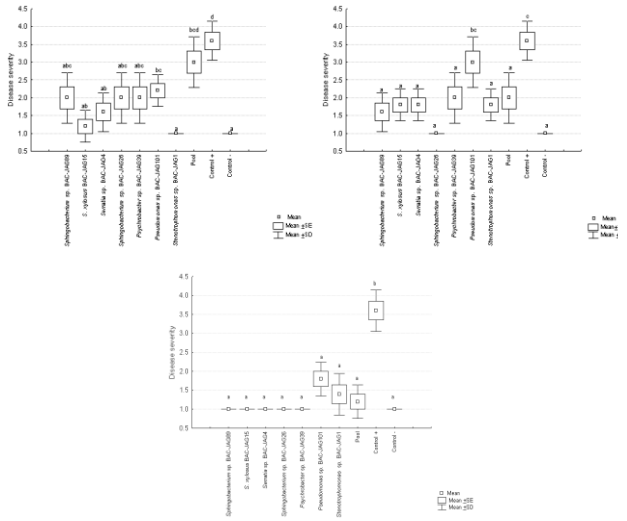

Fig. 6: Levels of vanilla stem damage before the presence of bacterial isolates in the three types of inoculation: a) stems immersed in bacterial suspension for 10 min; b) stems immersed in bacterial suspension for 10 minutes and inoculated three days after planting; c) stems inoculated three days after planting. Error bars represent the standard error of five replicates per sample. Different letters indicate significant differences (Wilcoxon test, $p<0.05$)

biocontrol agent as only some strains may be potentially harmful and act as opportunists in animal infections (Schleifer and Kloos, 1975; Dordet-Frisoni *et al.*, 2007). Some species of *Staphylococcus* possess antagonistic activities, such as *S. epidermidis* 2P3-18 isolated from the potato phyllosphere which presents some inhibition against *V. dahliae* and *R. solani* (Berg *et al.*, 2005; Kai *et al.*, 2007). *S. xylosus* BAC-JAG15 requires more evaluation, since even some strains of this species, as well as those of *Pseudomonas*, *Enterobacter*, *Azotobacter* and *Azospirillum* produce growth regulators such as ethylene, auxins and cytokinins, which can promote plant growth and induce systemic resistance against pathogens (Arshad and Frankenberger, 1991; Leifert *et al.*, 1994; Berg and Hallmann, 2006; Bhai and Kumar, 2008).

Another bacterial genus isolated from the roots of vanilla is *Stenotrophomonas*, its importance lies in its ecological functions within the cycles of elements in nature, its potential to promote plant growth and its applications in the biological control of plant fungal diseases (Ikemoto *et al.*, 1980; Berg *et al.*, 1994; Nakayama *et al.*, 1999; Kobayashi *et al.*, 2002). *Stenotrophomonas* sp. BAC-JAG1 showed antagonism against *Fov* in the three media tested (PDA 20%, NA 70% and Czapek 80%).

Similar results have been obtained in previous studies such as with *Stenotrophomonas rhizophila* and *S. maltophila* isolated from the rhizosphere of canola and potatoes showing antifungal properties, and the latter species showing high variability in its antifungal activity

(Berg *et al.*, 1999; Minkwitz and Berg, 2001; Wolf, 2002). It has been suggested that the mechanisms of inhibition of fungal isolates of the genus *Stenotrophomonas* are species dependent. These involve the production of secondary metabolites such as antibiotics and siderophores and enzymes to degrade the cell wall of fungi (proteases, glucanases and chitinases) (Minkwitz and Berg, 2001).

Apart from the genera described above, *Sphingobacterium* spp. BAC-JAG26 and BAC-JAG89 showed antifungal activity against *Fov* in inhibiting mycelial growth in two of the three media tested (PDA 52% and NA 80%). It is of interest to note that *Sphingobacterium multivorum* KST-009, from soil with added chitin, showed activity of a degrading enzyme for chitosan (chitosan SM1). Evaluation of germinated spores of *F. oxysporum* showed morphological changes such as protuberances in the hyphae which prevented cell differentiation at the ends of the hyphae and contributed to stopping or slowing mycelial elongation (Matsuda *et al.*, 2001). These results agree with those obtained by Sturz *et al.* (1999) who reported on the antifungal ability of the bacterial species *Sphingobacterium thalpophilum* and *Psychrobacter immobilis* against *Fusarium sambucinum*, *F. avenaceum* and *F. oxysporum* to slightly inhibit *in vitro* growth.

Although *Pseudomonas* species have been widely studied for their antifungal capacity, there are insufficient studies on this in vanilla. The work of Tombe *et al.* (1992) and Bhai and Kumar (2008) reported isolates of *Pseudomonas fluorescens* with *in vitro* activity against *Fov*. Tombe *et al.* (1997) included evaluations of greenhouse isolates of *P. fluorescens* which showed *in vitro* antifungal activity and concluded that these bacteria are effective at reducing the occurrence of rot when the stems or roots of vanilla were submerged in bacterial suspension before being transplanted into soil infested with *Fov*. Bhai and Kumar (2008) reported that *Fov* infection can be controlled by inoculating vanilla plants with isolates of *P. fluorescens* together with isolates of *Bacillus* sp. and *B. polymyxa*.

The results obtained in the present study clearly showed the differences in the quality and effectiveness of the bacterial isolates tested. The effect expressed by them in the greenhouse was different from that observed *in vitro*. These results agree with those reported by Knudsen *et al.* (1997) who noted that there is no correlation between the antagonism shown by bacterial isolates *in vitro* and those under greenhouse conditions. In the present work, most of the stems treated with bacterial inoculum initially developed symptoms of infection, but the disease did not advance, while in the control (cuttings without bacterial inoculum) infection spread from the root until the plant collapsed over a period of 30 days. These results demonstrate the protection offered by rhizobacteria at controlling *Fov* infection.

Cases such as *Psychrobacter* sp. isolate BAC-JAG39 had the lowest *in vitro* inhibition, had greenhouse antifungal properties in the three types of tested inoculations. The observed failure in controlling the antifungal activity observed in the greenhouse or the presence of chlorosis in vanilla stems may be due to problems with the procedure for proper establishment of bacterial isolates. It is known that environmental factors can result in poor distribution, insufficient development of strains in roots or poor expression of antagonistic activity by bacterial isolates (Duffy and Weller, 1995).

As well, some studies (Radjacommare *et al.*, 2010) have shown that care must be taken to generalize conclusions in such studies, since it is not known to what extent biocontrol activity will be affected by biotic factors (species and variety of plant, soil microbial activity), abiotic factors (soil type, water potential, soil temperature) and other factors (method and frequency of activities). Bhai and Kumar (2008) have emphasized that the degree of *in vitro* inhibition and the potential of rhizobacteria for suppression of *Fov* infection varies according to the type of insolate used.

The bacterial isolates used in this study were selected from culture media supplemented with chitin (crab shells). Future studies are needed to evaluate the antifungal activity of these isolates by adding the same ground chitin wastes or other sources of chitin, and verifying the effectiveness of biocontrol. Previous studies have indicated that application of *P. fluorescens* formulations with added chitin can raise the efficiency of antagonism to increase the capacity to degrade chitin in pathogen hyphae cell walls (Radjacommare *et al.*, 2010).

The greenhouse results show that all *in vitro* evaluations of biocontrol agents should be confirmed under field conditions, as there is no guarantee that candidates secreting toxins into the *in vitro* culture media and that cause pathogen inhibition will do so under field conditions. As the production of these secondary metabolites is known, results depend largely on the nutritional and specific environmental conditions (Köhl *et al.*, 2011), results that have been demonstrated under the *in vitro* conditions of the present study, showing differences in antagonism depend on the culture medium used.

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References

Adame-García, J., Á. Trigos, L.G. Iglesias-Andreu, N. Flores-Estevez and M. Luna-Rodríguez, 2011. Isozymic and pathogenic variations of *Fusarium* spp. associated with vanilla stem and root rotting. *Trop. Subtrop. Agroecosyst*., 13: 299–306

Arshad, M. and W.T. Frankenberger, 1991. Microbial production of plant hormones. *In: The Rhizosphere and Plant Growth*, pp: 327–334. D.L. Keister and P.B. Cregan (eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands

Åström, B. and B. Gerhardson, 1988. Differential reactions of wheat and pea genotypes to root inoculation with growth-affecting rhizosphere bacteria. *Plant Soil*, 109: 263–269

Beattie, G., 2006. Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. *In: Plant-Associated Bacteria*, pp: 1–56. S.S. Gnanamanickam (ed). Springer, The Netherlands

Berg, G., 2000. Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J. Appl. Microbiol*., 88: 952–960

Berg, G., 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol*., 84: 11–18

Berg, G. and J. Hallmann, 2006. Control of plant pathogenic fungi with bacterial endophytes. *In: Microbial Root Endophytes*, pp: 53–69. B.J.E. Schulz, C.J.C. Boyle and T.N. Sieber (eds.). Springer, Berlin, Heidelberg, Germany

Berg, G., C. Knaape, G. Ballin and D. Seidel, 1994. Biological control of *Verticillium dahliae* Kleb. by natural occurring rhizosphere bacteria. *Arch. Phytopathol. Plant Protect*., 29: 249–262

Berg, G., A. Krechel, M. Ditz, A. Faupel, A. Ulrich and J. Hallmann, 2005. Comparison of endophytic and ectophytic potato associated bacterial communities and their antagonistic activity against plant pathogenic fungi. *FEMS Microbiol. Ecol*., 51: 215–229

Berg, G., N. Roskot and K. Smalla, 1999. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. *J. Clin. Microbiol*., 37: 3594–3600

Bhai, R.S. and A. Kumar, 2008. Effect of rhizobacteria on *Phytophtora meadii*, *Fusarium oxysporum* f. sp. *vanillae* and *Colletotrichum vanillae* infecting vanilla. *J. Biol. Cont*., 22: 33–41

Bhai, R.S. and J. Thomas, 2000. Phytophthora rot -a new disease of vanilla (*Vanilla planifolia* Andrews) in India. *J. Spices Aromatic Crop*, 9: 73–75

Chang, W.T., C.S. Chen and S.L. Wang, 2003. An antifungal chitinase produced by *Bacillus cereus* with shrimp and crab shell powder as a carbon source. *Curr. Microbiol*., 47: 102–108

Chang, W.T., M.L. Chen and S.L. Wang, 2009. An antifungal chitinase produced by *Bacillus subtilis* using chitin waste as a carbon source. *World J. Microbiol. Biotechnol*., 26: 945–950

Cheng, H.R. and N. Jiang, 2006. Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol. Lett*., 28: 55–59

Dordet-Frisoni, E., G. Dorchies, C. De Araujo, R. Talon and S. Leroy, 2007. Genomic diversity in *Staphylococcus xylosus*. *Appl. Environ. Microbiol*., 73: 7199–7209

Duffy, B.K. and D.M. Weller, 1995. Use of Gaeumannomyces graminis var. graminis alone and in combination with fluorescent Pseudomonas spp. to suppress take-all of wheat. *Plant Dis.*, 79: 907–911

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783–791

Frankowski, J., M. Lorito, F. Scala, R. Schmid, G. Berg and H. Bahl, 2001. Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Arch. Microbiol*., 176: 421–426

Guevara-Avendaño, E., M. Luna-Rodríguez, P. Octavio-Aguilar, L.G. Iglesias-Andreu, Á. Trigos and M.J. Martínez-Hernández, 2014. Effect of rhizobacteria indole producing on the development of *Capsicum annuum* var. jalapeño M. *Int. Res. J. Biol. Sci*., 3: 22–27

Guzman, C.C., 2004. Vanilla. *In: Handbook of Herbs and Spices*, pp: 2–360. K. Peter (ed.). Woodhead Publishing Limited, England

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleid Acids Symp. Ser*., 41: 95–98

Hallmann, J., A. Quadt-Hallmann, W.F. Mahaffee and J.W. Kloepper, 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol*., 43: 895–914

Ikemoto, S., K. Suzuki, T. Kaneko and K. Komagata, 1980. Characterization of strains of *Pseudomonas maltophilia* which do not require methionine. *Int. J. Syst. Bacteriol*., 30: 437–447

Kai, M., U. Effmert, G. Berg and B. Piechulla, 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch. Microbiol*., 187: 351–360

Kalbe, C., P. Marten and G. Berg, 1996. Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiol. Res*., 151: 433–439

Kloepper, J.W., R. Rodríguez-Ubana, G.W. Zehnder, J.F. Murphy, E. Sikora and C. Fernández, 1999. Plant root-bacterial interactions in biological control of soilborne diseases and potential extension to systemic and foliar diseases. *Aust. Plant Pathol*., 28: 21–26

Kloepper, J.W. and M.N. Schroth, 1978. *Plant Growth Promoting Rizobacteria on Radishes*, Vol. 2, pp: 879–882. Proceedings of the fourth International Conference on Plant Pathogenic Bacteria, Station de Pathologie Végétale et Phytobactériologie, France

Kloepper, J.W., G. Wie and S. Tuzun, 1992. Rhizosphere population dynamics and internal colonization of cucumber by plant growth-promoting rhizobacteria which induce systemic resistance to *Colletotrichum orbiculare*. *In: Biological Control of Plant Diseases*, pp: 185–191. E.S. Tjamos, G.C. Papavizas and R.J. Cook (eds.). Springer, New York, USA

Knudsen, I.M.B., J. Hockenhull, D.F. Jensen, B. Gerhardson, M.Hökeberg, R. Tahvonen, E. Teperi, L. Sundheim and B. Henriksen, 1997. Selection of biological control agents for controlling soil and seed-borne diseases in the field. *Eur. J. Plant Pathol*., 103: 775–784

Kobayashi, D.Y., R.M. Reedy, J. Bick and P.V. Oudemans, 2002. Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. *Appl. Environ. Microbiol*., 68: 1047–1054

Köhl, J., J. Postma, P. Nicot, M. Ruocco and B. Blum, 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biol. Cont*., 57: 1–12

Leifert, C., C.E. Morris and W.M. Waites, 1994. Ecology of microbial saprophytes and pathogens in tissue culture and field-grown plants: reasons for contamination problems *in vitro*. *Crit. Rev. Plant Sci*., 13: 139–183

Luna, M., S. García, O. García and Á. Trigos, 2013. Serratin a new metabolite obtained from *Serratia marcescens*, a bacterium isolated from the microflora associated with banana plantations. *Nat. Prod. Res*., 27: 49–53

Matsuda, Y., Y. Iida, T. Shinogi, K. Kakutani, T. Nonomura and H. Toyoda, 2001. *In vitro* suppression of mycelial growth of *Fusarium oxysporum* by extracellular chitosanase of *Sphingobacterium multivorum* and cloning of the chitosanase gene *csn* SM1. *J. Gen. Plant Pathol*., 67: 318–324

Minkwitz, A. and G. Berg, 2001. Comparison of antifungal activities and 16S ribosomal DNA sequences of clinical and environmental isolates of *Stenotrophomonas maltophilia*. *J. Clin. Microbiol*., 39: 139–145

Minoo, D., V. Jayakumar, S. Veena, J. Vimala, A. Basha, K. Saji, K.N. Babu and K. Peter, 2008. Genetic variations and interrelationships in *Vanilla planifolia* and few related species as expressed by RAPD polymorphism. *Genet. Resour. Crop Evol*., 55: 459–470

Nakayama, T., Y. Homma, Y. Hashidoko, J. Mizutani and S. Tahara, 1999. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. *Appl. Environ. Microbiol*., 65: 4334–4339

Radjacommare, R., S. Venkatesan and R. Samiyappan, 2010. Biological control of phytopathogenic fungi of vanilla through lytic action of *Trichoderma* species and *Pseudomonas fluorescens*. *Arch. Phytopathol. Plant Prot*., 43: 1–17

Ribbeck-Busch, K., A. Roder, D. Hasse, W. De Boer, J.L. Martínez, M. Hagemann and G. Berg, 2005. A molecular biological protocol to distinguish potentially human pathogenic *Stenotrophomonas maltophilia* from plant-associated *Stenotrophomonas rhizophila*. *Environ. Microbiol*., 7: 1853–1858

Rovira, A.D., 1965. Interactions between plant roots and soil microorganisms. *Annu. Rev. Microbiol*., 19: 241–266

Schleifer, K.H. and W.E. Kloos, 1975. Isolation and characterization of *Staphylococci* from human skin. *Int. J. Syst. Bacteriol*., 25: 50–61

Sturz, A.V., B.R. Christie, B.G. Matheson, W.J. Arsenault and N.A. Buchanan, 1999. Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. *Plant Pathol*., 48: 360–369

Sturz, A.V. and J. Nowak, 2000. Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Appl. Soil Ecol*., 15: 183–190

Summerell, B.A., B. Salleh and J.F. Leslie, 2003. A utilitarian approach to *Fusarium* identification. *Plant Dis*., 87: 117–128

Talubnak, C. and K. Soytong, 2010. Biological control of vanilla anthracnose using *Emericella nidulans*. *J. Agric. Technol*., 6: 47–55

Tamura, K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*., 10: 512–526

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., 28: 2731–2739

Ting, A.S.Y., M.T. Fang and C.S. Tee, 2011. Efficacy of clay-based formulated *Serratia* in reducing inoculum of *Fusarium oxysporum* f. sp. *cubense* tropical race 4. *Acta Hortic*., 897: 421–426

Tombe, M., A. Nurawan, S.B. Nazarudin, K. Tsuchiya, M. Oniki and K. Matsumoto, 1992. Experiments on the introduction of biological and cultural control of stem rot disease of vanilla. *Ind. Crop Res. J*., 4: 20–26

Tombe, M., D. Sitepu and S. Mogi, 1997. Present status of biological control research of vanilla stem rot disease in Indonesia. *In: Plant Growth-Promoting Rhizobacteria-Present Status and Future Prospects*, pp: 13–21. A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, S. Akino (eds.). OECD, Sapporo, Japan

Wang, S.L., T.Y. Lin, Y.H. Yen, H.F. Liao and Y.J. Chen, 2006. Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. *Carbohydr. Res*., 341: 2507–2515

Wang, S.L., T.J. Chang and T.W. Liang, 2010. Conversion and degradation of shellfish wastes by *Serratia* sp. TKU016 fermentation for the production of enzymes and bioactive materials. *Biodegradation*, 21: 321–333

Wang, S.L., C.H. Chao, T.W. Liang and C.C. Chen, 2009. Purification and characterization of protease and chitinase from Bacillus cereus TKU006 and conversion of marine wastes by these enzymes. *Mar. Biotechnol.*, 11: 334–344

Welbaum, G.E., A.V. Sturz, Z. Dong and J. Nowak, 2004. Managing soil microorganisms to improve productivity of agro-ecosystems. *Crit. Rev. Plant Sci*., 23: 175–193

Weller, D.M., 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Ann. Rev. Phytopathol*., 26: 379–407

Wolf, A., 2002. *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. *Int. J. Syst. Evol. Microbiol*., 52: 1937–1944

Zachow, C., H. Pirker, C. Westendorf, R. Tilcher and G. Berg, 2009. The *Caenorhabditis elegans* assay: a tool to evaluate the pathogenic potential of bacterial biocontrol agents. *Eur. J. Plant Pathol*., 125: 367–376

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