SCIENTIFIC NOTE

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ENDOPHYTIC BACTERIA FROM *Pinus taeda* L. AS BIOCONTROL AGENTS OF *Fusarium circinatum* NIRENBERG & O'DONNELL

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Fusarium circinatum Nirenberg & O'Donnell, the pitch canker fungus, has been recently reported in Uruguay affecting *Pinus taeda* L. seedlings. The spread of this pathogen to plantations constitute a risk to forestry production. The aim of this work was to evaluate the inhibitory effect of live bacteria and their thermostable metabolites on *F. circinatum* growth *in vitro*. Four *Bacillus subtilis* strains and one of *Burkholderia* sp. isolated as *P. taeda* endophytes were evaluated as biological control agents of *F. circinatum*. Dual cultures between live bacteria and pathogen were performed. Furthermore, bacteria metabolites obtained from liquid cultures were sterilized and added to the culture media where fungus was grown. In this study all bacteria showed an antagonist effect on the pathogen growth arresting the mycelia at one cm of the edge of the bacteria colony. Bacteria thermostable metabolites reduced over 50% fungal growth. These results demonstrates that endophytic bacteria, well adapted to live in host tissues, constitute a good alternative to control *F. circinatum* affecting *Pinus* seedlings.

Key words: Biological control, *Bacillus* sp., *Burkholderia* sp., pitch canker.

The pitch canker fungus *Fusarium circinatum* Nirenberg and O´Donnell is a destructive pathogen that affects several *Pinus* species (Barnard and Blakeslee, 1980; Viljoen *et al*., 1994). The symptom more frequently associated to this pathogen is the presence of large resinous cankers on the main trunk and lateral branches of trees but it can also be associated to roots, shoots, cones, and seedlings. In plant seedlings aerial symptoms do not appear until the pathogen reach the trunk from lesions at soil level resulting in plant discoloration and needles drying. The disease has been detected in south eastern USA (Kuhlman *et al*., 1982), Mexico, South Africa, Chile, Japan, and Spain (Kobayashi and Muramoto, 1989; Guerra-Santos, 1999; Wingfield *et al*., 2002; Perez Sierra *et al*., 2007). Recently, in Uruguay this pathogen was detected on *Pinus taeda* L. seedlings from nurseries mainly affecting stem collar (Alonso and Bettucci, 2009).

According to Cook and Baker (1983) biological control can be defined as a reduction of the amount of inoculum or disease produced by the activity of a pathogen, based on the use of natural enemies or the use of compounds derived from its metabolism. Then, the biological control offers an alternative to the chemical products, contributing to minimize the negative consequences for human health and environment (Kim *et al*., 2003). Fungal diseases are very frequent in nurseries and the chemical control of pathogens is the most common practice.

Bacillus subtilis has been identified as a potent antagonist against several fungal pathogens due to the production of antifungal compounds, antibiotics and proteases, hence is extensively used in agricultural systems (Todorova and Kozhuharova, 2009; Chen *et al*., 2009; Kinsella *et al*., 2009).

Burkholderia sp. is known to have beneficial effect on plant growth through the production of antifungal and other compounds that are able to suppress many soil-borne plant pathogens (Holmes *et al*., 1998). *Burkholderia cepacia* is an ubiquitous soil organism that can be easily obtained and it has been studied as biocontrol agent of plant disease (Leisinger and Margraff, 1979). Many of its metabolites have been isolated and identified thus verifying its inhibitory effect on different plant pathogens such as fungus, bacteria and yeasts (Sophereth *et al*., 2006), particularly on species of *Pythium*, *Botrytis*, *Fusarium*, and *Rhizotocnia* (Sijam and Dikin, 2005; Quan *et al*., 2006).

The abuse and misuse of chemical products can cause environmental and human health-related risks. On the other hand, little work has been performed on biological control of forest pathogens. Identification and action mode of antifungal compounds produced by an antagonist need to be studied.

The aim of this work was to evaluate the antagonist effect of both live bacteria and their thermostable metabolites of four *Bacillus subtilis* strains and one of *Burkholderia* sp. on *Fusarium circinatum* growth.

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MATERIALS AND METHODS

Fungal and bacteria isolates

Fusarium circinatum strains used in this work were all isolated from symptomatic *Pinus taeda* seedlings from two *Pinus* nurseries from Rivera and Florida Departments in Uruguay. The fungal isolates were identified by macro and micromorphological characteristics and verified by molecular analysis using CIRC1A and CIRC4A specific primers for *F. circinatum* (Schweigkofler *et al*., 2004). The isolates were maintained in potato dextrose agar (PDA). Bacteria were present as endophytes from *Pinus* seedlings and were isolated from stem healthy tissues. Those showing inhibitory effect on fungal growth were selected. The identification of bacteria strains were performed by molecular analysis of 16S RNA region. The cultures were maintained on triptone soy agar (TSA).

Bacteria antagonist on *F. circinatum* growth

To evaluate the antagonist effect of different live bacteria, mycelia plugs from the edges of actively growing fungal cultures were placed in the center of Petri dish containing PDA. Four bacteria isolates were streaked on the same plates at equal distance from the fungal inocula. Plates with the fungal plug without bacteria were used as control. Plates were incubated at 25 °C for 5 d to evaluate the inhibition activity of bacteria on the fungus. Each treatment was replicated five times. The fungal strains used were Fc 2052, Fc2053, Fc2054, and Fc2057. Bacteria strains of *Bacillus subtilis* used were B1, B2, B3, B4, and one strain of *Burkholderia* sp. (B5).

Observations of mycelia of the interaction zone between fungi and bacteria were performed under microscope.

The activity of bacteria thermostable metabolites was also evaluated. Liquid cultures of bacteria were performed transferring colonies of each bacterium to a 250 mL Erlenmeyer flask containing 100 mL of potato dextrose broth (PDB) and then incubated in a rotary shaker at 27 °C and 180 rpm during 7 d. Ten milliliters of each flask were transferred to a new flask with 90 mL of PDA. These new flasks were sterilized during 16 min at 121 °C and 1 atm. The culture medium plus the metabolites were homogenized and 20 mL were placed on Petri dishes of 9 cm of diameter. Once the medium was solidified a plug of each fungal strain was placed in the centre of a dish. A fungal plug placed on PDA was used for control. Each treatment and control was replicated three times. Both treatments and controls were incubated at 25 °C during 9 d. After incubation for 120 h the diameter of the colonies was measured daily during 4 d and compared with controls. The measures were made from the centre of the fungal plug to the edge of the colony. Two measures were taken which were then averaged. Percentage of inhibition growth and the rate of growth were calculated. To determine if there were differences in the rate of growth between the four fungal strains tested a One Way ANOVA test was made using the Sigma Stat 3.5 program.

RESULTS AND DISCUSSION

Isolation of bacteria and screening of antifungal effect

The screening of bacteria for antifungal activity against the *Pinus* pathogen *F. circinatum* showed that all of them exhibited growth inhibition against the pathogen. All the strains arrested the mycelium growth at 1 cm or more of the fungal colony margin (Figure 1). The development of an inhibition halo was observed between the fungal colonies and the bacteria inocula. This may be due to the production of bacterial metabolites that may diffuse in the culture medium and suppress the growth of *F. circinatum*. These results are consistent with those obtained by Nourozian *et al*. (2006) who evaluated the antagonist activity of different bacteria (*Bacillus*, *Pseudomonas*) against *F. graminearum*. They observed, in dual culture experiments, the formation of inhibition zones between bacteria and fungus.

The micromorphology of mycelia in the interaction zone showed a change in hyphal mode development, exhibiting empty, vacuolated and swollen hypha and a different ramification pattern.

Effect of thermostable metabolites

Despite metabolites of all strains showed an inhibitory effect against the fungus strains tested (Figure 2) all of them had a different incidence on the fungal growth (Figure 3).

There were significant differences among the bacterial strains. Growth inhibition on the strain Fc2052 was greater than to the other strains ($p \leq 0.05$) (Figure 2). On the other hand, from all strains of *B. subtilis* the strain B2 showed the lowest effect on the pathogen growth. Although the fungal strain Fc2053 showed a lesser growth than the control, the difference was not significant. The effect of metabolites of *B. subtilis* B1 on Fc2054 was greater than to other fungal strains. Metabolites from *Burkholderia* sp. evidenced a lesser effect on the growth of this pathogen. Metabolites of B1, B3, B4, and B5 reduced the growth of *F. circinatum* Fc2057over 50% (Figure 2).

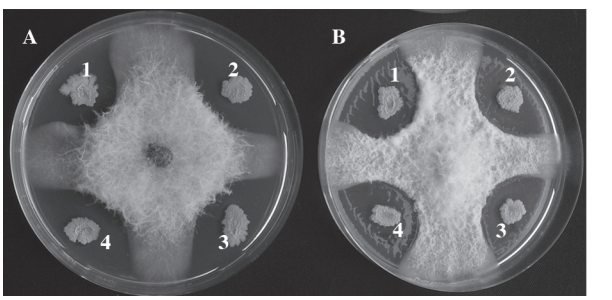

Figure 1. Inhibition halo produced on *Fusarium circinatum* strain FC2057 (A), and FC2053 (B) by *Bacillus subtilis* strains (1: B1; 2: B2; 3: B3; 4: B4).

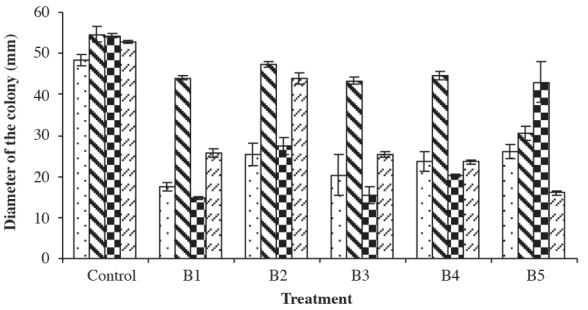

Figure 2. Antagonist effect of thermostable metabolites on *Fusarium circinatum* growth (±SD).
Growth of *Fusarium circinatum* strains Fc2052 □, Fc2053 ▨, Fc2054 ▩ and Fc2057 ▨ after 5 days on culture media containing thermostable metabolites of *Bacillus subtilis* strains (B1, B2, B3, B4) and *Burkholderia* sp. (B5). Control: culture media without metabolites.

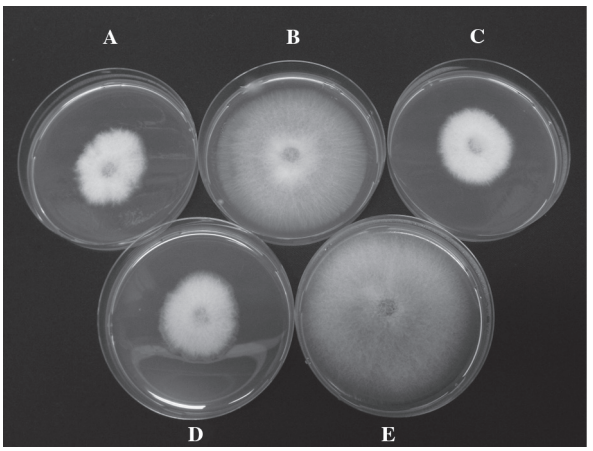

Figure 3. Growth of *Fusarium circinatum* strain Fc2057 on culture media containing thermostable metabolites of *Bacillus subtilis* strains (A: strain B1; B: strain B2; C: strain B3; D: strain B4; E: control, culture media without metabolites).

These results showed that the metabolites of *Bacillus* and *Burkholderia* tested, reduced the rate of growth of *F. circinatum* although some differences among fungal strains were observed. These findings suggest the possibility of using *B. subtilis* as biocontrol agent of *Pinus* pathogen *F. circinatum*, consistently with other studies where *Bacillus* has inhibitory effect against *Fusarium* spp. and other plant pathogen fungi (Moita *et al*., 2005; Kinsella *et al*., 2009). Recently, *Burkholderia* spp. have been used as biocontrol agents against fungal disease, including *Fusarium* spp. (Quan *et al*., 2006).

CONCLUSIONS

The bacteria that were present as endophytes of *Pinus taeda* seedlings were symptomless colonizers and apparently adapted to host tissues. This can constitute an advantage for using them as biocontrol agent of the pitch canker fungus on this host. The biological control could be an alternative to reduce the incidence of the pathogen in nurseries in order to avoid the expansion of the disease to the field. The active thermostable metabolites are also a very interesting alternative to chemical control and to avoid the use of living organisms. Both, *B. subtilis* and *Burkholderia* are not frequently used in forest management.

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Bacterias endófitas de *Pinus taeda* L. como agentes de control biológico de *Fusarium circinatum* Nirenberg & O'Donnell. La presencia de *Fusarium circinatum* Niremberg & O´Donnell, agente causal del cancro resinoso en pino, ha sido detectada recientemente en plántulas de *Pinus taeda* L. en Uruguay. La propagación de este patógeno en las plantaciones constituye un riesgo para la producción forestal. El objetivo de este trabajo fue determinar la capacidad inhibitoria de bacterias vivas y de sus metabolitos termoestables sobre el crecimiento de *F. circinatum in vitro*. Cuatro cepas de *Bacillus subtilis* y una de *Burkholderia* sp. aisladas como endófitas de *P. taeda*, fueron evaluadas como potenciales agentes de control biológico sobre *F. circinatum*. Para ello, se realizaron enfrentamientos directos entre las bacterias vivas y el micelio del patógeno. Por otra parte, los metabolitos bacterianos obtenidos de cultivos líquidos fueron esterilizados en autoclave y se incorporaron al medio de cultivo donde se hizo crecer el patógeno. En este estudio todas las bacterias mostraron un efecto antagónico sobre el crecimiento del patógeno, deteniéndose el avance del micelio a 1 cm del borde de la colonia bacteriana. Los metabolitos termoestables de las bacterias produjeron una disminución significativa en la tasa de crecimiento del hongo mayor al 50%. Estos resultados muestran que las bacterias que viven dentro de los tejidos sanos del hospedante son una buena alternativa para el control del patógeno *F. circinatum* en plántulas de *Pinus*.

Palabras clave: control biológico, *Bacillus* sp., *Burkholderia* sp., cancro resinoso.

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