Growth promotion of *Pinus radiata* seedlings by soil inoculation and seed pretreatment with the biological control agent *Clonostachys rosea*

Promoción de crecimiento de plántulas de *Pinus radiata* a través de aplicación del agente de control biológico *Clonostachys rosea* en suelo y pretratamiento de semillas

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ABSTRACT

Twenty-one *Clonostachys rosea* strains, previously selected by its biocontrol activity higher than 80% against *Fusarium circinatum*, were evaluated for its ability to promote growth of *Pinus radiata* seedlings on natural substrate. Stem height, root length, dry weight of stem and roots, and ectomycorrhization were measured at 102 days after seeding. The results showed significant differences between some of the tested *C. rosea* strains when compared to the controls, showing increases for plant height of 25%, dry stem mass of 37%, root length of 13.5%, dry root mass of 20.5% and ectomycorrhization of 55% when compared to their respective controls, demonstrating a possible dual use of these strains for both plant protection and increased biomass of *P. radiata*.

KEYWORDS: Biocontrol agents, biofertilizers, growth promoters, nurseries.

INTRODUCTION

*Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (teleomorph *Bionectria ochrouleuca*) is a non-pathogenic and cosmopolitan fungus with a worldwide distribution and also recognized as mycoparasite (Hoopen et al. 2003, Lübeck et al. 2002, Sutton et al. 2002). The antagonist activity of *C. rosea* is of wide spectrum, and is currently recognized as a strong biological control agent (BCA) against several pathogenic fungi (Lahoz et al. 2004, Morandi et al. 2001, Nobre et al. 2005, Rodriguez et al. 2011, Sutton et al. 2002, Tarantino et al. 2006). Studies aimed to the evaluation of *C. rosea* against important diseases in the forestry such as the gray mold caused by *Botrytis cinerea* Pers. on seedlings of *Eucalyptus globulus* Labill. (Molina et al. 2006, Zaldúa & Sanfuentes 2010), and the damping-off caused by *Fusarium circinatum* Nirenberg & O’Donnell on *Pinus radiata* D. Don (Moraga-Suazo et al. 2011, Moraga-Suazo et al. 2016), demonstrated a reduction on the disease by diverse mechanisms, reaching biocontrol levels higher than 80% (Valdebenito 2016).

The plant growth promotion effect has been observed on inoculated plants with *C. rosea* on different crops, including wheat, tomato, barley, rose, geranium and cucumber (Johansen et al. 2005, Ravnskov et al. 2006, Roberti et al. 2008, Sutton et al. 2008). In forest species there are few...
cases of growth promotion stimulated by microorganisms, as an example, in poplar hybrids (Populus trichocarpa × Populus deltoides) growing on sandy soils, an endophytic bacterium isolated from the stems, Enterobacter sp. strain 638, increased the biomass production on cuttings in greenhouse assays (Taghavi et al. 2009).

Currently there is no information on the effect of C. rosea strains with biocontrol activity, on the growth promotion of forest species, therefore the aim of this study was to evaluate the effect of selected C. rosea strains as growth promoters in P. radiata seedlings.

MATERIALS AND METHODS

PLANT MATERIAL AND C. ROSEA STRAINS
Seeds from P. radiata originated from controlled pollinations, were provided by Forestal Mininco S.A. Twenty-one C. rosea strains belonging to the collection of Forest Pathology Lab at University of Concepción were included in this study (Table 1). The strains were collected mainly from root and soil of P. radiata plantations and selected for their activity as BCA, providing protection over 80% against F. circinatum under greenhouse conditions (Valdebenito 2016). C. rosea strains were stored in tubes containing Potato Dextrose Agar (PDA) as culture medium at 4 ºC and replicated in Petri dishes containing PDA and growth at 25 ºC for 7d, this inoculum was used to produce large-scale cultures of the strains by solid media cultivation according to Cavalcante et al. (2008).

GROWTH PROMOTION ASSAYS
Conidial suspension (1x10⁷ conidia x mL⁻¹ in water) of each C. rosea strain was applied by immersion of seeds for 30 min, 24 h before seeding. A second C. rosea application to the natural substrate was applied at seeding time and a third application after germination, in both cases by spraying the substrate. Non-sterilized (natural) composted pine cortex substrate was used for the assay. A commercial product (Trichonativa®, Bioinsumos Nativa Ltda, Chile) based on Trichoderma spp. was used as control treatment (CT). In order to compare to the tested strains, the active ingredient concentration was adjusted to 1x10⁷ conidia x mL⁻¹. Sterilized distilled water was used as an absolute control treatment (AT). Eighty-four seeds for each treatment, with three replicates by treatment and twenty-three treatments were established. The experiment was carried out under operational conditions in the Carlos Douglas nursery belonging to Forestal Mininco S.A. At 102 days after seeding, forty plants were collected from the center of each tray in order to evaluate phenotypical characteristics, separating the root system from the substrate with water. A caliper was used to measure the stem height and root length. Roots and stems were then blotted dry with paper towels, separated into root and stem sections, and then dried at 105 ºC for 48 h in order to measure the dried weight. The percentage of ectomycorrhizal colonization was estimated by counting the number of root tips with ectomycorrhizal colonization (visualized as short roots) over the total number of root tips obtained for each seedling (Walbert et al. 2010).

DATA ANALYSIS
The experiment was arranged in a completely randomized design with twenty-three treatments. Each treatment was composed of three replicates and each replicate consisted of thirty seedlings in order to evaluate height and dry weight of stem, and length and dry weight of root. For the percentage of ectomycorrhizal colonization, the five treatments with best performance in growth promotion were selected. Each treatment was composed of three replicates and ten seedlings per replicate. Statistical data analysis was performed by ANOVA at a significance level of 0.05. All data were subjected to analysis of homogeneity of variance and normality assumptions and pooled accordingly. Multiple comparisons were made using a Tukey test. Analyses were performed with statistical analysis software SAS (SAS Institute 2000).

RESULTS AND DISCUSSION
The mean height for absolute control treatment (AT) was 6.8 cm, eight treatments (T1, T2, T3, T11, T12, T15, T16 and T17) showed mean heights significantly higher than AT, with T16 presenting the highest height of 8.5 cm, an increment of 25% when compared to AT and also higher than commercial treatment (CT = 7.2 cm, Fig. 1A). About stem biomass, nine treatments (T1, T2, T3, T5, T9, T11, T12, T15 and T16) showed differences when compared to AT, with T1 presenting the highest value of 0.26 g, representing an increment of 37% in biomass when was compared with AT (0.19 g). CT (0.20 g) was not different to AT (Fig. 1B).

Five strains (T7, T12, T13, T14 and T17) increased length of roots when compared to AT (Fig. 2A), T14 presented the highest value of 12 cm, which increased 13.5% with respect to AT (10.6 cm). No significant differences were found between AT and CT treatments. For root dry weight, four treatments (T1, T8, T12 and T16) presented significant differences with AT, the highest value for root dry biomass was obtained by T12 (0.182 g), representing an increment of 20.5% higher than AT (0.151 g). For root biomass CT control was not significantly different to AT (Fig. 2B).

As indicated on Figure 3, T2 (45%) and T3 (51%) treatments showed percentages of ectomycorrhizal colonization higher than AT (32.9%), showing potential for increasing plant productivity and root quality by the use of selected C. rosea strains, which will allow a better performance of the antagonist-treated plants from nurseries to their final plantation field.
Sutton et al. (2008) reported that the application of Pg 88-710 strain of C. rosea increased growth and production of roses, geraniums and cucumbers. The same beneficial effect was found by Ravnskov et al. (2006) in tomato plants, showing an increase in the content of phosphorus and a reduction of nitrogen content in leaves after antagonist application. Application of antagonists also increased the weight of plants 30 days after sowing (Macedo 2011). In our study, several strains were able to promote growth, significantly increasing stem height (25%), stem dry weight (37%), root length (13.5%), root dry weight (20.5%) and ectomycorrhizal colonization (55%). Even when several strains do not increase the growth, none of the isolates showed a deleterious effect on plants.

BCAs may have an important role on plant growth promotion, but the information for forest species is still scarce. Donoso et al. (2008) demonstrated the effect of Trichoderma harzianum Rifai applied on pine cortex substrate in the growth promotion of P. radiata, increasing the height, total biomass and aerial biomass of treated plants. In the same pine species, the application of T. harzianum along with ectomycorrhizal fungi Suillus luteus (L.) Roussel and Rhizopogon luteolus Fr. stimulated plant growth (Chavez et al. 2014). Nevertheless, to date the few studies of strains of C. rosea on forest species were focused on the biocontrol effect against the pathogens Botrytis cinerea (Zaldúa & Sanfuentes 2010) and F. circinatum (Moraga-Suazo et al. 2011, Valdebenito 2016, Moraga-Suazo et al. 2016). Even when the biocontrol effect was successful, there is a lack of information about the potential growth effect of these BCAs over forest nurseries.

Table 1. Information of each treatment employed in this study. / Información de cada tratamiento empleado en este estudio.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Place Name</th>
<th>Species</th>
<th>Tissue/Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>T00</td>
<td>Absolut treatment: water</td>
<td></td>
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</tr>
<tr>
<td>T01</td>
<td>Carlos Douglas</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T02</td>
<td>Los Castaños</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T03</td>
<td>Quivolgo</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T04</td>
<td>La Posada</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T05</td>
<td>Quivolgo</td>
<td>Pinus radiata</td>
<td>Shoot</td>
</tr>
<tr>
<td>T06</td>
<td>La Posada</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T07</td>
<td>Fundo El Sauce</td>
<td>Agronomic Crop</td>
<td>Soil</td>
</tr>
<tr>
<td>T08</td>
<td>San Isidro</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T09</td>
<td>La Posada</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T10</td>
<td>Quillón</td>
<td>Vineyard</td>
<td>Soil</td>
</tr>
<tr>
<td>T11</td>
<td>Los Castaños</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T12</td>
<td>La Posada</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T13</td>
<td>Ruta Itata</td>
<td>Populus sp.</td>
<td>Soil</td>
</tr>
<tr>
<td>T14</td>
<td>Coyanmahuida</td>
<td>Native Forest</td>
<td>Soil</td>
</tr>
<tr>
<td>T15</td>
<td>Santa Juana</td>
<td>Agronomic Crop</td>
<td>Soil</td>
</tr>
<tr>
<td>T16</td>
<td>San Isidro</td>
<td>Pinus radiata</td>
<td>Root</td>
</tr>
<tr>
<td>T17</td>
<td>La Quila</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T18</td>
<td>Coyanmahuida</td>
<td>Non identified</td>
<td>Shoot</td>
</tr>
<tr>
<td>T19</td>
<td>Quivolgo</td>
<td>Pinus radiata</td>
<td>Soil</td>
</tr>
<tr>
<td>T20</td>
<td>Parral</td>
<td>Agronomic Crop</td>
<td>Soil</td>
</tr>
<tr>
<td>T21</td>
<td>Quillón</td>
<td>Maytenus boaria</td>
<td>Shoot</td>
</tr>
<tr>
<td>T22</td>
<td>Control treatment: comercial product (Trichoderma harzianum, T. virens, T. parceramosum)</td>
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Correa et al. (2010) studied the *C. rosea* performance applied to lettuce in a hydroponic cultivation system and observed that *C. rosea* was able to reduce the incidence of root rot caused by *Pythium aphanidermatum* (Edson) Fitzp., nevertheless in this case the antagonist failed to promote plant growth, indicating that the interaction between antagonists (strains) and host could be specific for each case (Moreira et al. 2014). This results indicate that not always an antagonistic strain has both good biological control and growth promoting activity, showing the requirement to study both characteristics for each selected strain.

The present study was conducted in natural substrate, indicating that the seedlings response to the evaluated *C. rosea* strains corresponds to a direct interaction of the *C. rosea* strains with the host tissues, rather than an indirect interaction of pathogen suppression. It is worth to note that the strains employed in this study were mainly isolated from *P. radiata* soil and roots, this could be a key factor in the success performance of some strains because a better adaptation to the introduced environment and microbial competition. It is not clear what is the effect of *C. rosea* with the microorganisms present in the rhizosphere, or with the ectomycorrhizal species associated with *P. radiata*. There are three possible outcomes between mycorrhizal fungi and BCA or growth promoters: synergy (Fracchia et al. 2003, Martinez et al. 2004), antagonism (Ravnskov et al. 2006) and neutrality (Mar Vasquez et al. 2000, Siddiqui and Akhtar 2009). In the case of the recognized BCA *T. harzianum*, also mentioned as a growth promoter, different effects on mycorrhizal fungi have been found, being synergistic to *Glomus intraradices* N.C. Schenck & G.S. Sm. in melon plants (Martinez-Medina et al. 2010), neutral to *G. deserticola* Trappe, Bloss & J.A. Menge in maize plants (Mar Vasquez et al. 2000), and antagonistic for *G. intraradices* in cucumber plants (Green et al. 1999).

In the present study, a synergistic effect generated by the T2 and T3 was observed, while the other treatments (T1, T12, T16, T17) generated a neutral effect. This contrasts with a previous study where an antagonist effect between *C. rosea* and *G. intraradices* was observed (Ravnskov et al. 2006).

According to these records it is possible to indicate that the effect of *C. rosea* on roots of the host seems to be dependent on the particular strain tested.
Based on these results, it is clear that the strains with both increased growth promotion of *P. radiata* and improvement effect on their root quality, have potential as inoculants on the forest industry. All *C. rosea* strains tested here were selected due to their biocontrol capacity against *F. circinatum* higher than 80% on operational conditions on nurseries of *P. radiata*, and will be included for the future formulation of biological products aimed at the improvement of the sanitary status (control of *F. circinatum*) and the increase of the yields of *P. radiata*.

**Figure 2.** Effect of *Clonostachys rosea* applied as seed pretreatment and soil on length and dry weight of roots. Asterisks indicate treatments with means significantly higher than absolute control treatment (AT) (*p*=0.05 Tukey test). Vertical bars correspond to standard error, *n* = 30 plants per treatment. A, Mean length (cm) per treatment. B, Mean dry weights (g) per treatment. / Efecto de *Clonostachys rosea* aplicado como pretratamiento de semillas y suelo sobre la longitud y peso seco de raíces. Asteriscos indican tratamientos con medias significativamente más altas que el control absoluto (*p*=0.05, test de Tukey). Barra vertical corresponde a error estándar, *n*=30 plantas por tratamiento, 3 repeticiones. A: Longitud media (cm) por tratamiento. B: Peso seco promedio (g) por tratamiento.

**Figure 3.** Effect of *Clonostachys rosea* applied as seed pretreatment and soil inoculation on percentage of ectomycorrhization. Asterisks indicate treatments with means significantly higher than absolute control treatment (AT) (*p*=0.05 Tukey test). Vertical bar correspond to standard error, *n* = 10 seedlings per treatment, 3 replicates. / Efecto de *Clonostachys rosea* aplicado como pretratamiento de semillas y suelo sobre el porcentaje de ectomicorrización. Asteriscos indican tratamientos con media significativamente mayor que el control absoluto (AT) (*p*=0.05, test de Tukey). Barra vertical corresponde a error estándar, *n*=10 plantas por tratamiento, 3 réplicas.
CONCLUSION

C. rosea strains enhance the health of P. radiata seedlings and increase their growth, along with an improvement of root quality increasing the percentage of ectomycorrhized roots.

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