Gayana Bot. 74(1): 140-146, 2017 ISSN 0016-5301
Original Article

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.

RESUMEN

Veintiún aislamientos de *C. rosea*, previamente seleccionadas por su actividad de biocontrol superior al 80% contra *Fusarium circinatum*, fueron evaluadas en su habilidad para promover el crecimiento de plántulas de *Pinus radiata* sobre substrato natural. Las variables altura de tallo, longitud de raíz, masa seca de tallo y raíz, y ectomicorrización fueron medidas a los 102 días posterior a la siembra. Los resultados muestran el aumento significativo de las variables evaluadas con la aplicación de algunos de los aislamientos de *C. rosea*, alcanzando incrementos del 25% en altura, 37% en peso seco del tallo, 13,5% en la longitud de la raíz, 20,5% en peso seco de la raíz, y 55% de ectomicorrización, demostrando un posible uso dual de estas cepas tanto para protección fitosanitaria como para incremento en la biomasa de *P. radiata*.

Palabras clave: Agentes de biocontrol, biofertilizantes, promotores de crecimiento, viveros.

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 x 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 ($1x10^7$ 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 $1x10^7$ 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.

Treatment	Place Name	Species	Tissue/soil
T00	Absolut treatment: water		
T01	Carlos Douglas	*Pinus radiata*	Soil
T02	Los Castaños	*Pinus radiata*	Root
T03	Quivolgo	*Pinus radiata*	Soil
T04	La Posada	*Pinus radiata*	Soil
T05	Quivolgo	*Pinus radiata*	Shoot
T06	La Posada	*Pinus radiata*	Root
T07	Fundo El Sauce	Agronomic Crop	Soil
T08	San Isidro	*Pinus radiata*	Root
T09	La Posada	*Pinus radiata*	Soil
T10	Quillón	Vineyard	Soil
T11	Los Castaños	*Pinus radiata*	Root
T12	La Posada	*Pinus radiata*	Root
T13	Ruta Itata	*Populus* sp.	Soil
T14	Coyanmahuida	Native Forest	Soil
T15	Santa Juana	Agronomic Crop	Soil
T16	San Isidro	*Pinus radiata*	Root
T17	La Quila	*Pinus radiata*	Soil
T18	Coyanmahuida	Non identified	Shoot
T19	Quivolgo	*Pinus radiata*	Soil
T20	Parral	Agronomic Crop	Soil
T21	Quillón	*Maytenus boaria*	Shoot
T22	Control treatment: comercial product (*Trichoderma harzianum, T. virens, T. parceramosum*)		

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. Schenk & 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.

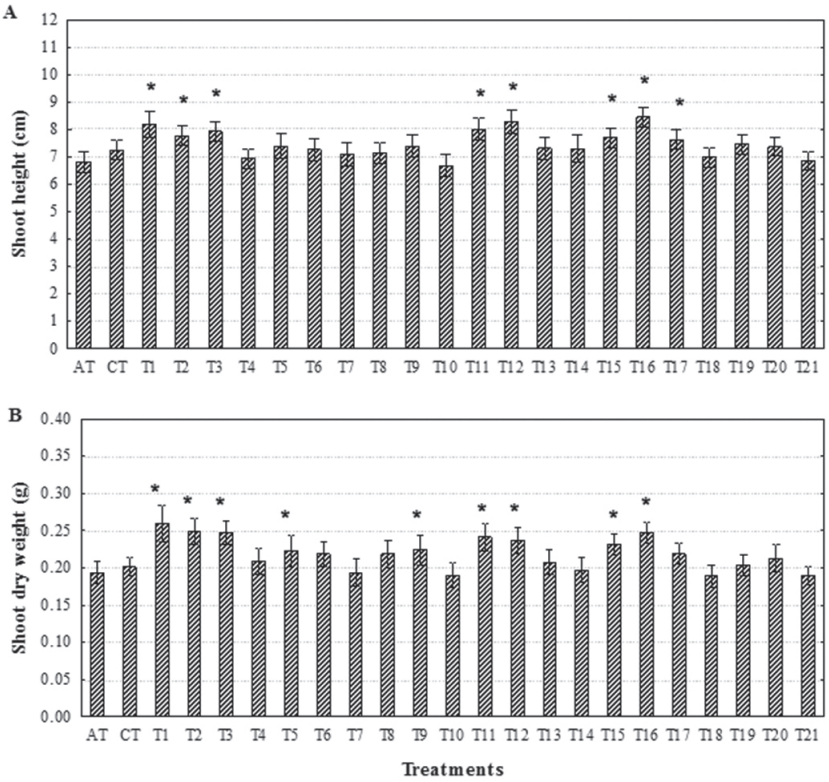

Figure 1. Effect of *Clonostachys rosea* applied as seed pretreatment and soil on height and dry weight of shoots. Asterisks indicate treatments with means significantly higher than absolute control treatment (AT) (p=0.05 Tukey test). Vertical bars correspond to standard error, n = 30 plants per treatment. A, Mean height (cm) per treatment. B, Mean dry weight (g) per treatment. / Efecto de la aplicación de *Clonostachys rosea* como pretratamiento de semillas y suelo sobre la altura y peso seco de brotes. 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: Altura media (cm) por tratamiento. B: Peso seco promedio (g) por tratamiento.

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*.

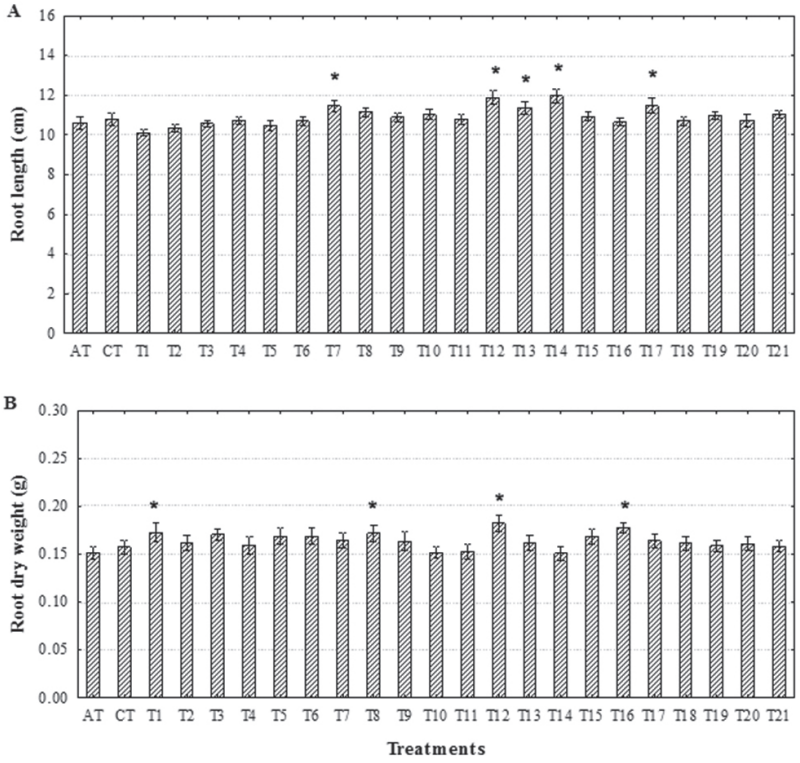

Figure 2. Effect of *Clonostachys rosea* applied as seed pretreatment and soil on length and dry weight of roots. Asterisks indicate treatments with means significantly higher than absolute control treatment (AT) (p=0.05 Tukey test). Vertical bars corresponds to standard error, n = 30 plants per treatment. 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.

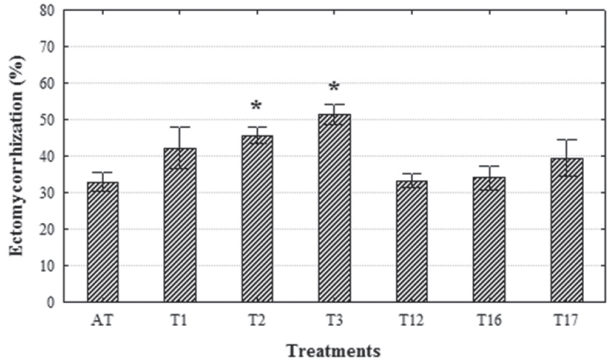

Figure 3. Effect of *Clonostachys rosea* applied as seed pretreatment and soil inoculation on percentage of ectomycorrhization. Asterisks indicate treatments with means significantly higher than absolute control treatment (AT) (p=0.05 Tukey test). Vertical bar correspond to standard error, n = 10 seedlings 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.

ACKNOWLEDGEMENTS

The authors acknowledge Mr Francisco Rodríguez for provide plant material and facilities on Forestal Mininco S.A. This work was funded by Postdoctoral Fellowship FONDECYT Nº 3130606.

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Recibido: 24.05.2016
Aceptado: 13.03.2017