The fungus *Fusarium circinatum* Nirenberg & O'Donnell causes pine pitch canker, an important disease for conifers worldwide. *F. circinatum* was first detected in Chile in 2001 and to date is present in nurseries and clonal hedges from Libertador General Bernardo O’Higgins Region to Los Ríos Region. The purpose of this study was to evaluate the potential of *Trichoderma* spp. and *Clonostachys* spp. strains to control *F. circinatum* in *Pinus radiata* D. Don seedlings in the absence of other effective control methods. Eighty-one *Trichoderma* spp. and *Clonostachys* spp. strains were evaluated through *in vitro* assays to determine their ability to act as antagonists of *F. circinatum* and 21 strains were tested for their ability to reduce post-emergence mortality and increase *P. radiata* survival under greenhouse conditions. During *in vitro* experiments, 15 strains of *Trichoderma* inhibited mycelial growth of the pathogen by more than 60% and one strain of *Clonostachys* showed parasitism of *F. circinatum* hyphae. Greenhouse experiments showed no control of the disease when the antagonists were added to substrate after the pathogen. However, when the antagonists were added before the pathogen, four strains (*Clonostachys* UDC-32 and UDC-222 and *Trichoderma* UDC-23 and UDC-408) reduced post-emergence mortality between 80 and 100%. Among these strains, only *Clonostachys* UDC-222 significantly increased the survival of *P. radiata* seedlings. These results showed that *Clonostachys* UDC-222 has the potential to be used as a biocontrol agent against *F. circinatum* in the production of *P. radiata* plants.

**Key words:** Antagonists, biological control, seedling death, pitch canker.

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with other measures, that reduce the use of pesticides has become increasingly important. Antagonistic microorganisms, including fungi and bacteria, applied as treatments for seeds provide unique and beneficial opportunities for the production of different crops, especially protection against soil-borne pathogenic fungi (Mao et al., 1997).

Several studies have used fungi and bacteria to control diseases caused by Fusarium species affecting different crops (Mao et al., 1997; Mao et al., 1998; Bacon et al., 2001), including some non-pathogenic strains of Fusarium spp. (Paulitz et al., 1987; Postma and Rattink., 1992; Larkin and Fravel, 2002; Silva and Bettiol, 2005). Nonetheless, few studies have been done on biological control of F. circinatum, among them the use of Trichoderma harzianum strains to control the disease in P. patula seedlings (Mitchell et al., 2005).

Despite the importance of the disease, no effective methods for its control have been found, no chemical products have been registered and no biological agents being marketed. This limits disease management options in affected nurseries. Due to current and potential damage arising from the presence of F. circinatum, the secondary dissemination risk in plantations and the lack of efficient control methods, the objective of this study was to select and evaluate strains of Trichoderma spp. and Clonostachys spp. for the control of F. circinatum in P. radiata seedlings.

**MATERIALS AND METHODS**

**Antagonists and pathogen strains**

Strains of Trichoderma (71 strains) and Clonostachys (10 strains) were selected for the assays from the Forest Pathology Laboratory collection from the Universidad de Concepción. These strains have shown activity as antagonists against other pathogens in previous studies (Grandón, 2006; Mellado, 2006; Molina et al., 2006). One F. circinatum strain (Pr44-4641) was also included, isolated from symptomatic P. radiata hedges that had more aggressive behavior in previous pathogenicity tests (González, 2005). Antagonistic fungi and pathogens were stored in tubes containing potato dextrose agar (PDA) as culture medium at 4 °C. Fungal strains were replicated in Petri dishes containing PDA and incubated at 25 °C for 2 to 3 d. The paired cultures were incubated at 25 °C for 7 d before being assessed. The pathogen plug was inoculated 24 h before the antagonist plug on each Petri dish. After the assay, the mycelial growth inhibition (MGI) of F. circinatum was determined along with the presence of parasitism in the pathogen hyphal.

The MGI was obtained by dividing the pathogen colony area into different dual cultures facing the antagonists by the pathogen colony area from the control treatment. Colony surface areas were measured with a dot grid of 0.5 × 0.5 cm.

Parasitism was determined under microscope observation at 100X and 400X, observing the interaction area between colonies confronting each other in the dual cultures. The occurrence of coiled hyphae was considered to be evidence of hyphal parasitism. When no interactions were clearly observed among hyphae, new pairings were made on slides coated with a thin film of agar-water (AW) and incubated at 25 °C for 2 to 3 d.

**Control of F. circinatum in P. radiata seedlings**

Eighteen strains of Trichoderma and three of Clonostachys were used for this study. These strains were selected during an earlier in vitro antagonism test; since they showed either higher pathogen mycelial inhibition or hyphal parasitism, and some of these selected strains have also been used to control other pathogens such as Macrophomina phaseolina and Botrytis cinerea (Grandón, 2006; Molina et al., 2006). The experiments were performed in the greenhouse propriety CPF (Controladora de Plagas Forestales) and authorized by the government agency Servicio Agrícola y Ganadero (SAG) for quarantine tests with F. circinatum.

Pinus radiata seeds provided by Forestal Mininco S.A, previously surface disinfected for 15 min with hydrogen peroxide, were sown in trays containing pine bark compost previously sterilized by autoclaving twice at 121 °C for 30 min, with an interval of 24 h. Two assays were established simultaneously: in the first assay, the antagonists were applied 7 d before F. circinatum inoculation, and in the second assay, the pathogen was inoculated 48 h before antagonists. The substrate was sprayed with separate suspensions containing each antagonist (10^7 to 10^8 conidia mL^-1) and pathogen (10^3 conidia mL^-1) in sterile distilled water.

Two comparative treatments were used in both assays. In the first treatment, only the pathogen was applied and in the second one, only water was applied as a control. The assays were maintained for 60 d after seedling emergence and two variables were evaluated, post-emergence mortality and seedling survival.
Post-emergence mortality was defined as those seedlings having the symptoms described for damping-off. The post-emergence mortality rate was obtained by dividing the number of dead seedlings by the total seedlings emerged (%). Emerged seedlings were considered as those with cotyledons above the substrate surface. All dead seedlings were collected, surface disinfected with sodium hypochlorite (5% v/v) for 3 min, and then transferred to Petri dishes with PDA medium to determine infection by *F. circinatum*.

The seedling survival rate was obtained by dividing the number of symptom-free *P. radiata* seedlings at the end of the assay by the number of seeds sown (%).

**Experimental design and data analysis**

The *in vitro* studies used a completely randomized design with four replicates for each strain plus a control (*F. circinatum* only). The experimental unit consisted of one Petri dish. Greenhouse experiments also used a completely randomized design with four replicates, and the experimental unit was composed of five plastic containers with one seedling each. Statistical data analysis was performed by ANOVA with a significance level of 0.05. All the data were subjected to analysis of homogeneity of variance and normality assumptions and pooled accordingly. Multiple comparisons were made using the Tukey test. Analyses were performed with Statistical Analysis System software (SAS Institute, 2000).

**RESULTS AND DISCUSSION**

**In vitro antagonistic activity**

The *in vitro* assays showed mainly an inhibitory effect of the antagonistic strains on the growth of *F. circinatum* colony (Figure 1). Many of the *Trichoderma* strains colonized a large area of the culture medium in the plates due to the speed of their mycelial growth, which was higher than that of *F. circinatum*. On the contrary, *Clonostachys* strains normally had slower growth rates than the pathogen. Furthermore, it was also noted that several *Trichoderma* strains sporulated abundantly when growing over the pathogenic colony, thereby indicating that they can be highly competitive for space and nutrients (Figure 1B). Only *Clonostachys* UDC-222 (Figure 1C) was capable of parasitizing *F. circinatum* hyphae (Figures 2A and 2B). None of the tested strains showed antagonism by antibiosis.

From the 81 strains assayed *in vitro*, 76 significantly reduced *F. circinatum* mycelial growth. Of these, 49 inhibited the pathogen by 40 to 60% and 15 by 60 to 68%. All strains inhibiting the mycelial growth belonged to the genus *Trichoderma*. Strains of *Clonostachys* showed lower levels of pathogen inhibition (1-23%), among them *Clonostachys* UDC-A32 had the highest value (Figure 3).

These results are consistent with previous studies in which *Trichoderma* strains have been reported to be agents that inhibit pathogen growth as a biocontrol mechanism (Benítez *et al*., 1997). The species also produces fungal inhibitors and bodies in various types of fungi is well known (Sutton *et al*., 1997). From the 81 strains assayed *in vitro*, 76 significantly reduced *F. circinatum* mycelial growth. Of these, 49 inhibited the pathogen by 40 to 60% and 15 by 60 to 68%. All strains inhibiting the mycelial growth belonged to the genus *Trichoderma*. Strains of *Clonostachys* showed lower levels of pathogen inhibition (1-23%), among them *Clonostachys* UDC-A32 had the highest value (Figure 3).

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enzymes (glucanases) that degrade cell walls, inducing the loss of turgor and causing lysis of pathogenic hyphae (Papavizas, 1985; Sutton et al., 1997).

**Control of *F. circinatum* in *P. radiata* seedlings**

When the pathogen was added to substrate 48 h before antagonists, none of the variables evaluated differed significantly between treatments, with post-emergence mortality of seedlings ranging from 25% to 100%. Only three *Trichoderma* strains (UDC-280, UDC-351, UDC-404) showed less than 50% seedlings mortality. However, these had low survival, indicating that much of the mortality occurred before the seedlings emerged. In the control treatment (without the application of the pathogen), the post-emergence mortality was high (89%). This was attributed to the presence of the inoculant in the greenhouse environment, which allowed the pathogen to colonize the treatment, mainly targeting the aerial parts of plants through the cotyledons (data not shown).

When the antagonists were added to the substrate before adding the pathogen, significant differences were observed in post-emergence mortality and seedling survival (Figures 4 and 5). The application of *Trichoderma* strains UDC-23 and UDC-408 significantly reduced post-emergence mortality, with values of 8.3% and 0%, respectively, compared to 94% mortality obtained when applying *F. circinatum* only. On the other hand, *Clonostachys* strains UDC-A32 and UDC-222 reduced post-emergence mortality to 20.8% and 8.3%, respectively (Figure 4).

Regarding the survival of seedlings, the four strains able to reduce post-emergence mortality were the same ones that had the highest values of seedling survival. However, a significant increase was achieved in this variable (69%) by applying *C. rosea* UDC-222. Treatments in which only the pathogen was added had seedling survival rates of about 5% (Figure 5).

The performance of *C. rosea* UDC-222 in reducing post-emergence mortality and increasing seedling survival indicates that this fungus is able to protect seedlings from the beginning of the development of (pre-emergence stage), producing a protective effect on the seed. The better capacity of *C. rosea* UDC-222 to act as a biocontrol agent could be explained in part by its use of two forms of antagonism: competition for space and nutrients and, more importantly, parasitism of the hyphae. Considering that both the pathogen and the antagonist presented similar growth rates, this form of antagonism collapsed pathogenic hyphae, removing it from the substrate and previously colonized tissues (Sutton et al., 1997).

Another hypothesis that could explain the success of this strain is its ability to colonize. Some *Clonostachys* strains are known to act as non-pathogenic endophytes, colonizing host tissues without causing alterations in the plant and thereby eliminating the potential development and sporulation of pathogenic fungal agents (Sutton et al., 2002). In this case, *C. rosea* UDC-222 could have colonized some seedling tissues before the arrival of the pathogen, allowing a reduction in the incidence of the disease.

This study has demonstrated the importance of timing in the application of biocontrol agents: good results were obtained only when the antagonists were previously incorporated in the substrate. This strategy was also demonstrated in studies with raspberries, where *C. rosea* suppressed sporulation and interfered with infection and colonization of *B. cinerea* most effectively when applied before or when the pathogen was introduced (Yu and Sutton, 1997).

**CONCLUSION**

These results indicate that *C. rosea* UDC-222 has the ability to reduce the incidence of *F. circinatum* in *P. radiata* seedlings, establishing the potential use of antagonists for the control of this pathogen in forestry nurseries. However, initial results indicate that control is only possible before the pathogen reaches the substrate.

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González, G. 2005. Resultados preliminares del estudio In vitro antagonism de B. bacon, J., I. Yates, D. Hinton, and M. Filmore. 2001. Biological control de Clonostachys spp. para controlar Fusarium circinatum en plantas de Pinus radiata. Fusarium circinatum Nirenberg & O’Donnell es el hongo que causa el cancro resinoso del pino, una enfermedad de importancia mundial en coníferas. En Chile, F. circinatum fue detectado por primera vez el año 2001 y a la fecha se encuentra presente en algunos viveros y huertos clonales desde la Región del Libertador General Bernardo O’Higgins hasta la Región de Los Ríos. Debido a la ausencia de métodos eficientes para controlar este patógeno, el propósito de este estudio fue evaluar el potencial uso de cepas de Trichoderma y Clonostachys como control de Fusarium circinatum en plantas de Pinus radiata. D. Don. Ochenta y un cepas de Trichoderma spp. y Clonostachys spp. fueron evaluadas a través de ensayos in vitro para determinar su habilidad para actuar como antagonistas de Fusarium circinatum y 21 cepas fueron probadas por su habilidad para reducir la mortalidad post-emergencia e incrementar la supervivencia de P. radiata durante condiciones de invernadero. Durante los experimentos in vitro, 15 cepas de Trichoderma inhibieron el crecimiento del patógeno más de un 60% y una cepa de Clonostachys mostró parasitismo de hifas de F. circinatum. En experimentos en invernadero, los antagonistas no controlaron la enfermedad cuando fueron agregados después de la inoculación del patógeno. Sin embargo, cuando los antagonistas fueron sembrados antes del patógeno, cuatro cepas (Clonostachys UDC-32 y UDC-222, Trichoderma UDC-23 y UDC-408) redujeron la mortalidad post-emergencia entre 80 y 100%. Entre estas cepas, sólo Clonostachys UDC-222 incrementó significativamente la supervivencia de plantas de P. radiata. Estos resultados demuestran que Clonostachys UDC-222 posee gran potencial para ser empleado como agente de biocontrol contra Fusarium circinatum en la producción de plantas de P. radiata.

Palabras clave: antagonistas, control biológico, muerte de plantas, pitch canker.

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