

CONTROL OF *Botrytis cinerea* IN *Eucalyptus globulus* MINI-CUTTINGS USING *Clonostachys* AND *Trichoderma* STRAINS

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ABSTRACT

Botrytis cinerea Pers. ex Fr. causes the disease known as gray mold in more than 200 hosts. It is one of the most important pathogens in Chilean forest nurseries and *Eucalyptus globulus* Labill. is one of the most susceptible species, especially in vegetative reproduction systems. *Clonostachys* and *Trichoderma* strains were selected as potential biocontrol agents of gray mold in previous research by the authors. The objective of this study was to evaluate the effectiveness of antagonistic fungi to control *B. cinerea* in *E. globulus* mini-cuttings. Five fungi strains were tested and applied weekly, two *Clonostachys* and three *Trichoderma* (5×10^6 conidia mL⁻¹). In addition, comparison treatments were also used: absolute control (water) and fungicide application. The experiment was carried out under operational conditions to produce *E. globulus* mini-cuttings. The *Clonostachys* UDC-A10 and UDC-A11 strains reduce mini-cutting mortality caused by *B. cinerea* in 54 and 71%, respectively, and with effects similar to those achieved by fungicides. *Clonostachys* UDC-A11 reduces the disease progression rate with the same statistical results as fungicides. A negative effect of applying fungicides on rooting of the surviving mini-cuttings was also confirmed. These results demonstrate the effectiveness of *Clonostachys* as a control agent against gray mold disease in *E. globulus* mini-cuttings.

Key words: biocontrol agents, forest nurseries, eucalyptus, gray mold, antagonistic fungi.

INTRODUCTION

The *Botrytis cinerea* Pers. ex Fr. pathogen fungi causes gray mold disease in a wide variety of hosts (Coley-Smith *et al.*, 1980; Elad *et al.*, 2004), and is a serious economic problem in crops such as table grapes, vines, strawberries, raspberries, lettuce, cucumbers, broad beans, tomatoes, beans, flowers, and forest plants produced in containers (Elad *et al.*, 2004).

Gray mold is an important sanitary problem in *Pinus radiata* D. Don and *Eucalyptus globulus* Labill. nurseries in Chile, and is especially severe in the latter species. Problems caused by *B. cinerea* have been observed in the majority of forest nurseries in the Southern Hemisphere (Butin and Peredo, 1986) with different disease incidence and severity levels, causing losses greater than 50% in some cases. In addition, in the last few years, it has caused

a serious rotting problem in cuttings associated with high relative humidity and temperature which are conditions required in this plant production system.

Numerous research studies in Canada, the United States, Israel, and Brazil have shown the potential and effectiveness of microorganisms to control *B. cinerea*, mainly *Clonostachys* and *Trichoderma* strains, both in greenhouse and field tests, and obtaining better results in some cases than for recommended fungicides (Sutton and Peng, 1993; Zhang *et al.*, 1994; Elad *et al.*, 1996; Sutton *et al.*, 1997; Sanfuentes and Ferreira, 1997; Wilson, 1997; Yu and Sutton, 1997; Gerlagh *et al.*, 2001; Nobre *et al.*, 2005; Elmer and Reglinski, 2006; Cota *et al.*, 2008).

The possibility of using antagonistic microorganisms in Chile to control *B. cinerea* has been mainly studied in agricultural crops with *Trichoderma* strains (Esterio and Auger, 2005; Harman *et al.*, 1996; Latorre *et al.*, 1997). On the other hand, a few research studies have been done using this control strategy in forest nurseries (Molina *et al.*, 2006). Given the importance of the disease in Chile and the inherent problems to control it, the objective of this study was to evaluate the effectiveness of *Clonostachys* and *Trichoderma* strains to control *B. cinerea* in *E. globulus* mini-cuttings.

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MATERIALS AND METHODS

Plant material and experimental conditions

The experiment was performed at La Posada nursery, Bosques Arauco S.A. (Coronel Road, km 16.5, Bío Bío Region (36°55'33" S, 73°9'10" W). *Eucalyptus globulus* clonal mini-cuttings (5-7 cm) were collected from hydroponic hedges to which no fungicide was applied 1 wk before the experiment was established.

The experiment was carried out in a greenhouse with automatic nebulizers to maintain high relative humidity (70-100%) and an optimal temperature range (20-28 °C) for mini-cutting rooting in accordance with the company's plant production program. The experiment lasted 45 d between the end of January and the beginning of March 2006. Dead plants were periodically extracted during the experiment, in accordance with the company's operational control norms.

Inoculum production

Antagonist strains were transferred to Petri dishes or test tubes with potato dextrose agar medium (PDA, Difco BD, Maryland, USA) and incubated for 15-20 d at 21-24 °C with a photoperiod of 12:12 h to produce inoculum. Conidia were collected in sterile distilled water (SDW). Concentration was adjusted with the help of a hemacytometer (Superior, West Germany).

Treatments

Five fungi strains were tested, four previously selected as effective antagonists for *B. cinerea* (Molina *et al.*, 2006), i.e., two *Clonostachys* (UDC-A10 and UDC-A11) and two *Trichoderma* (UDC-A27 y UDC-A6), and the fifth was a *Trichoderma* (EF-1) strain provided by the company. Moreover, two comparison treatments were used: an operational fungicide treatment in the nursery and a control treatment, which consisted in applying only water (corresponding to natural inoculum/infection of pathogen).

Mini-cuttings were submerged in antagonist suspensions for 1 min in 1×10^7 conidia mL⁻¹ concentration immediately after collection. The fungicide treatment included immersion in cyprodinil + fludioxonil (Switch 62,5 WG, 37.5% + 25.0% respectively, Syngenta Agribusiness, Basel, Switzerland) (3 g L^{-1}), and mini-cuttings were maintained in water for the same period of time in the control treatment. Mini-cuttings were placed in the operational substrate of vermiculite and perlite (1:1). Then, antagonists were applied weekly by sprinkling mini-cuttings and watering the substrate at a rate of 5×10^6 conidia mL⁻¹. Fungicides used in the sprinklers were selected and applied according to the company's operational gray mold control program, and was applied

as follows: Switch, captan (Captan 83 WP, BASF Chile, Santiago, Chile), fenhexamid (Teldor 50% WP, Bayer CropScience AG, Monheim am Rhein, Germany), iprodione (Rovral 50% WP, Bayer CropScience A.G.), and Switch measured at 3 g L^{-1} of the commercial product.

A total of five treatments were applied during the experiment on 25 January, 1, 8, 15, and 22 February according to the company's operational gray mold control program. Sprinkling antagonists (200 mL per tray) and fungicides (250 mL per tray) was done with a manual sprinkler. The substrate was watered only for the antagonist treatments, 5 mL per mini-cutting with a syringe.

Evaluations

Disease incidence was evaluated during the experiment by removing dead mini-cuttings every 3 d (typical stem necrosis) which were deposited in paper bags and maintained at 4 °C. These materials were checked in the Forest Pathology Laboratory with a stereomicroscope (SR, Zeiss, West Germany) in order to verify the presence of *B. cinerea*. Mini-cuttings without any signs of gray mold were taken to a moist chamber to stimulate sporulation and verify the diagnosis.

In the final evaluation of the experiment, all of the plants were collected, and both *B. cinerea* incidence and mini-cutting survival were determined. Disease incidence was calculated as the percentage of dead mini-cuttings to total number analyzed in the treatment. Additionally, rooting of the remaining mini-cuttings was evaluated and expressed as the percentage of the total of surviving mini-cuttings. Mini-cuttings were considered as rooted if they had roots equal or longer than 3 cm at the time of evaluation.

Experimental design and data analysis

The experiment was conducted in a randomized complete block design with six replications. An experimental plot consisted of a tray with 88 mini-cuttings, and only 54 central mini-cuttings were considered for the analysis.

Exponential, monomolecular, logistic, Gompertz, and linear models were adjusted to analyze the development of disease incidence over time (Table 1). The model was selected by considering the coefficient of determination (R^2), mean square for error (MSE), and standard deviation (Campbell and Madden, 1990). Once the best model was obtained, disease progression rates (r_{π}) were compared among all curves, and in all of the possible pair combinations.

When the data did not meet normality assumptions (Shapiro-Wilk Test) and variation homogeneity (Bartlett Test) for the statistical analysis of final disease incidence and rooting variables, they were thus transformed using

Table 1. Models used to analyze the progress of gray mold in *Eucalyptus globulus* mini-cuttings where y : final inoculum, y_0 : initial inoculum, r_i : disease progress rates, and t : time.

Model name	Linearized model
Exponential	$\ln(y) = \ln(y_0) + r_E \cdot t$
Monomolecular	$\ln\left(\frac{1}{1-y}\right) = \ln\left(\frac{1}{1-y_0}\right) + r_M \cdot t$
Logistic	$\ln\left(\frac{y}{1-y}\right) = \ln\left(\frac{y_0}{1-y_0}\right) + r_L \cdot t$
Gompertz	$-\ln[-\ln(y)] = -\ln[-\ln(y_0)] + r_G \cdot t$
Linear	$y = y_0 + r_i \cdot t$

squared root to fix the problem. All data were analyzed by ANOVA and multiple comparisons by the Least Significant Difference (LSD) Test at $\alpha = 0.05$. Analyses were performed by the Statistical Analysis System program (SAS Institute, 2002) and Statistica (StatSoft, 2007).

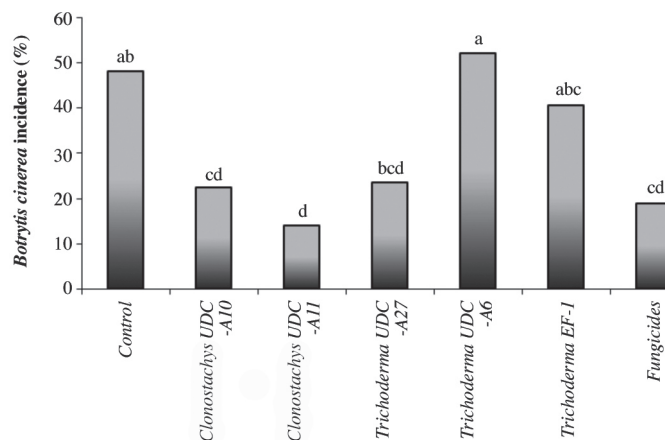
RESULTS

According to the symptoms observed in the mini-cuttings, the most frequent place of *B. cinerea* infection was in the zone adjacent to the stem cut in contact with the substrate where the pathogen colonized tissues and advanced towards the apex of the mini-cutting. Affected mini-cuttings were also observed in the first vertical leaf and in the apex, but this represented only a few cases. It was common to observe mini-cuttings attacked by *B. cinerea* forming callus and/or roots.

Disease incidence and progression rate

At the end of the experiment, *B. cinerea* incidence in *E. globulus* mini-cuttings showed significant differences among treatments ($p = 0.01$). The lowest *B. cinerea* incidence was obtained by applying *Clonostachys* UDC-A11 (14%) and the highest disease incidence was with *Trichoderma* UDC-A6 (52%) (Figure 1). The *Clonostachys* UDC-A10 and UDC-A11 strains significantly reduced *B. cinerea* incidence with control levels of 54 and 71%, respectively, and had a statistical effect similar to fungicides (61%). Applying *Trichoderma* UDC-A6, UDC-A27, and EF-1 resulted statistically similar to the control, with the latter two achieving control levels of 51 and 16%, respectively. The atypical increase of *B. cinerea* incidence at the end of the experiment (Figure 2) was attributed to the fact that some of the mini-cuttings with gray mold symptoms had not been detected during the partial recording of the disease and continued in the experiment until the final evaluation when all the mini-cuttings were harvested.

Disease progression was adjusted in all treatments to the exponential model (Table 1) and showed the best adjustment parameters: coefficient of determination (R^2), MSE, and standard deviation. The highest disease progression rate was obtained with the control treatment, and *Trichoderma* UDC-A6 and EF-1. The comparison between disease progression rates (r_E) (Table 2) showed that the control treatment disease progression rate was statistically different from all other treatments, except *Trichoderma* EF-1 ($p = 0.1441$). This strain was also statistically similar to *Trichoderma* UDC-A6 ($p = 0.4760$). Disease progression rates for *Clonostachys* UDC-A10 and *Trichoderma* UDC-A27 were statically similar ($p = 0.7136$) and lower than the control. Applying *Clonostachys* UDC-A11 and fungicides significantly reduced the disease progression rate which is statistically similar ($p = 0.1630$)



Different letters indicate significant differences according to LSD Test ($\alpha = 0.05$).

Figure 1. *Botrytis cinerea* incidence in *Eucalyptus globulus* mini-cuttings at the end of the experiment.

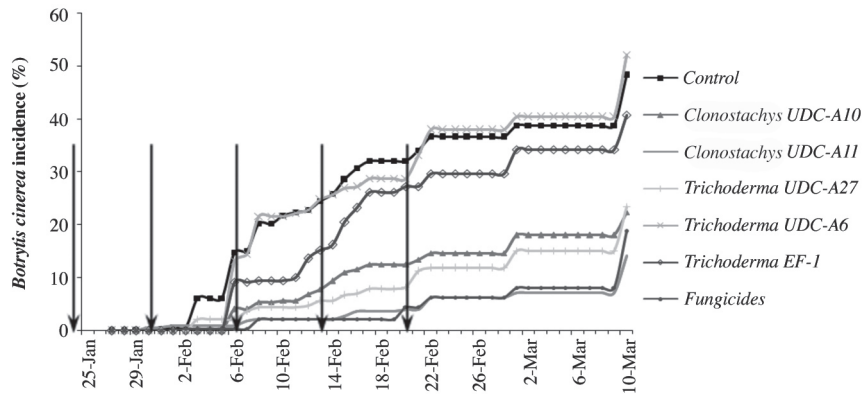


Figure 2. Evolution of *Botrytis cinerea* incidence in *Eucalyptus globulus* mini-cuttings during the experiment. Arrows indicate the application of antagonists and fungicides.

in both treatments. These two treatments exhibited similar mini-cutting mortality behavior with the lowest disease incidence and progression rate during the experiment.

Mini-cutting rooting

Significant differences in mini-cutting rooting between treatments were verified ($p < 0.0001$). All antagonist treatments showed no significant differences with the control (80% rooting). By applying *Clonostachys* UDC-A10 and UDC-A11, as well as *Trichoderma* UDC-A27, the highest levels of rooting fluctuated between 88 and 90% which is significantly different from fungicides (Figure 3). The fungicide treatment exhibited low rooting levels with a reduction of 87%, which was significantly lower than the control and the rest of the applied antagonists.

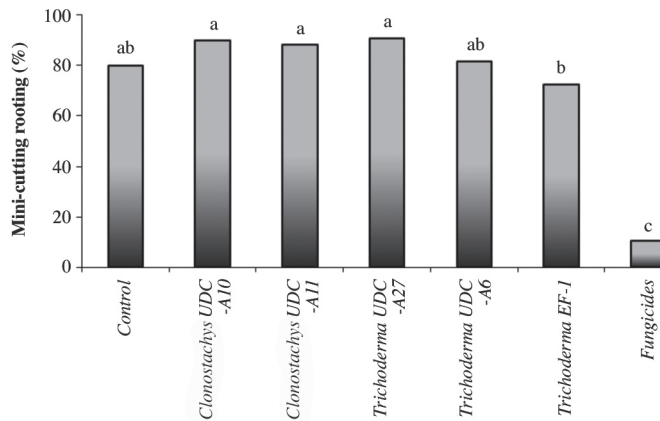
DISCUSSION

The *Clonostachys* UDC-A10 and UDC-A11 strains reduced *B. cinerea* incidence with respect to the control treatment, and obtained control levels of 54 and 71%, respectively. Comparing the effectiveness of control attained by these antagonists and results obtained in other research studies, a moderate to high control effect is possible (Sutton *et al.*, 2002) since there was a naturally high disease pressure during this experiment. Control attained by the antagonists could be attributed to their arriving before the pathogen to plant tissues (Sutton and Peng, 1993; O'Neill *et al.*, 1996) since they were applied immediately after collecting the mini-cuttings. The *Clonostachys* species action mechanisms have not been completely studied, but it is thought that mycoparasitism, substrate competition, antibiosis, and induced systematic resistance play an important role (Sutton *et al.*, 1997). In addition, it has been shown that *C. rosea* can penetrate without causing damage or deterioration in the host's tissues and act as an endophyte (Sutton and Peng, 1993;

Table 2. Values for initial inoculum (y_0) and disease progression rate (r_T) of *Botrytis cinerea* during the experiment with *Eucalyptus globulus* mini-cuttings adjusted according to the exponential model for all treatments.

Variable	Standard deviation	Coefficient*
Control		
$R^2 = 0.5128$ CME = 1.34528		
y_0	0.13885	-4.37154
r_{T0}	0.00544	0.09129b
<i>Clonostachys</i> UDC-A10		
$R^2 = 0.3979$ CME = 1.25100		
y_0	0.13390	-4.84787
r_{T1}	0.00524	0.06975c
<i>Clonostachys</i> UDC-A11		
$R^2 = 0.2642$ CME = 0.89195		
y_0	0.11306	-4.67697
r_{T2}	0.00443	0.04342d
<i>Trichoderma</i> UDC-A27		
$R^2 = 0.4861$ CME = 0.93881		
y_0	0.11599	-4.82267
r_{T3}	0.00454	0.07229c
<i>Trichoderma</i> UDC-A6		
$R^2 = 0.6684$ CME = 0.95238		
y_0	0.11683	-4.69751
r_{T4}	0.00457	0.10629a
<i>Trichoderma</i> EF-1		
$R^2 = 0.6485$ CME = 0.95211		
y_0	0.11681	-4.85050
r_{T5}	0.00457	0.10168ab
Fungicides		
$R^2 = 0.3785$ CME = 0.74863		
y_0	0.10358	-4.88637
r_{T6}	0.00405	0.05180d

*Different letters indicate significant differences between disease progression rates ($\alpha = 0.05$).



Different letters indicate significant differences according to LSD test ($\alpha = 0.05$).

Figure 3. Rooting of *Eucalyptus globulus* mini-cuttings in the greenhouse after 45 d.

Zhang *et al.*, 1996; Yu and Sutton, 1997; Morandi *et al.*, 2000), thus giving it an advantage over other biological control agents, such as some bacteria and filamentous fungi which remain on the surface of the plant and must fight against the destructive influences of the phylloplane's micro-climate. Plant production from cuttings requires constant water nebulization which can result in the loss of antagonists on the phylloplane, and leave the tissues unprotected against *B. cinerea*. This may be the reason for which *Trichoderma* strains were less effective in controlling the disease, unlike *Clonostachys* strains which could protect tissues from the inside and reach high control levels. The same *Trichoderma* strains (UDC-A6 and UDC-A27) had better results controlling the same pathogen under different environmental conditions (Molina *et al.*, 2006). Different studies are currently being conducted to explain which antagonism mechanisms are involved in the tested *Clonostachys* strains.

Adjusting the exponential model to *B. cinerea* incidence in this experiment means that there is a directly proportional relationship between the disease progression rate and disease incidence, and an indirect relationship with the initial inoculum (Campbell and Madden, 1990) (Figure 2). The effect that certain antagonists had in reducing the disease progression rate indicates that biocontrol agents directly affect *B. cinerea* sporulation and reduce secondary inoculum (secondary infections). This result also verifies the importance of the secondary inoculum in this pathosystem, especially since environmental conditions in this experiment were highly favorable to the development of the disease.

In the production of forest plants from seeds, *B. cinerea* typically attacks the apical portion (young tissue or damaged by abiotic factors) or aging leaves attached to the stem (Butin and Peredo, 1986). However, the pathogen

in this experiment was observed as mainly attacking the cut zone of mini-cuttings in contact with the substrate and directly affected root development. It is necessary to analyze the control strategy in this pathosystem given this different way of attacking by emphasizing antagonist protection in the stem (neck) and in callus formation zone to better protect the plant, as well as complementing it with traditional strategies to protect the aerial part of the seedlings.

The *Clonostachys* UDC-A10 and UDC-A11 and *Trichoderma* UDC-A27 strains obtained higher mini-cutting rooting levels. Certain specific plant-microorganism interactions can positively influence plant growth through a series of mechanisms in the case of rooting. These effects could be attributed to an increase in tolerance to a different abiotic stress, controlling pathogens, and/or directly or indirectly promoting plant growth and development (Bais *et al.*, 2006). Similar results were observed in research by Oliveira *et al.* (2007) and Donoso *et al.* (2008) who used *Trichoderma* strains to promote plant growth and rooting of *P. radiata* and *Eucalyptus* sp., respectively. The same effect was observed in miniature roses, geraniums, and cucumbers (plants and cuttings) treated with *C. rosea* which accelerated rooting of the cuttings, increased plant height and root dry mass, accompanied with disease reduction (Sutton *et al.*, 2008). On the other hand, the treatment applying fungicides in this experiment achieved one of the best mini-cutting survival levels. However, there was a negative effect on root formation with only 10% rooting. The mini-cuttings in this treatment remained vigorous due to the formation of an abundant undifferentiated tissue mass (callus). Perhaps this means that the rhizogenesis process was delayed rather than root formation being inhibited. A similar effect appeared in geranium stems treated with

C. rosea and fungicides where the antagonist developed abundant, healthy, and more vigorous roots than those treated with fungicides (Sutton *et al.*, 1997). The effect of antagonists or fungicides on rooting should be accurately established in future tests.

CONCLUSION

Clonostachys UDC-A10 and UDC-A11 strains were effective in reducing mini-cutting mortality caused by *B. cinerea* during the rooting phase. Moreover, *Clonostachys* UDC-A11 reduces the disease progression rate with statistical effects similar to those achieved by fungicides. In addition, the greatest rooting values were obtained for mini-cuttings treated with *Clonostachys* and *Trichoderma* strains, and applying fungicide had a negative effect on the control treatment.

These results indicate the effectiveness of *Clonostachys* as a control agent against gray mold in the production of *E. globulus* mini-cuttings.

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RESUMEN

Control de *Botrytis cinerea* en miniestacas de *Eucalyptus globulus* utilizando cepas de *Clonostachys* y *Trichoderma*. *Botrytis cinerea* Pers. ex Fr. ocasiona la enfermedad conocida como moho gris en más de 200 hospederos. En Chile es uno de los patógenos más importantes en viveros forestales, siendo *Eucalyptus globulus* Labill. una de las especies más susceptibles, especialmente en los sistemas de reproducción vegetativa. En investigaciones previas, realizadas por los autores, se seleccionaron cepas de *Clonostachys* y *Trichoderma* como potenciales agentes de biocontrol del moho gris. El objetivo fue evaluar la eficacia de hongos antagonistas en el control de *B. cinerea* en mini-estacas de *E. globulus*. Se probaron cinco cepas de hongos, dos de *Clonostachys* y tres de *Trichoderma* (5×10^6 conidias mL⁻¹) aplicados semanalmente. Además, se utilizaron tratamientos para realizar comparaciones: control absoluto (agua) y aplicación de fungicidas. El ensayo se realizó bajo condiciones operacionales de producción de mini-estacas de *E. globulus*. Las cepas de *Clonostachys* UDC-A10 y UDC-A11 redujeron la mortalidad de las mini-estacas

ocasionada por *B. cinerea* en 54% y 71%, respectivamente, con efectos similares a los logrados por los fungicidas. *Clonostachys* UDC-A11 redujo la tasa de incremento de la enfermedad con resultados estadísticamente similares a los obtenidos con fungicidas. Además, con la aplicación de fungicidas se constató un efecto negativo en el enraizamiento de las mini-estacas sobrevivientes. Estos resultados demuestran la eficacia de *Clonostachys* como agente de control contra la enfermedad del moho gris en mini-estacas de *E. globulus*.

Palabras clave: agentes de biocontrol, eucalipto, viveros forestales, moho gris, hongos antagonistas.

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