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ORIGINAL ARTICLE

Clonostachys rosea: effect of additives on its efficiency in inhibiting *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs.

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ABSTRACT

Musiet, D.; Sanfuentes, E.; Sossa, K.; Bettiol, W. *Clonostachys rosea*: effect of additives on its efficiency in inhibiting *Botrytis cinerea* sporulation on eucalyptus leaf discs. v.51, p.1-7, 2025.

The present study was carried out to evaluate: the effects of additives and their mixtures, used in pesticide formulations, on the conidial germination of *Clonostachys rosea*, and the efficacy of *Clonostachys* strains A10 and A11, alone or in mixture of additives, in inhibiting *Botrytis cinerea* conidial sporulation on *Eucalyptus globulus* leaf discs at different temperatures. Conidial germination of *Clonostachys* strains in potassium and calcium carbonate (0.5, 1, 2 g L^{-1}), cornstarch and sodium alginate (10, 15, 20 g L^{-1}) was similar to that of the control with water (93.7% − 97.3%); for titanium dioxide (2, 4, 6 g L^{-1}) and carboxymethylcellulose (0.5, 1, 2 g L^{-1}), the inhibition was between 7% and 16%. Glycerol completely inhibited conidial germination. The viability of *Clonostachys* conidia was maintained in mixtures containing additives (potassium and calcium carbonate, titanium dioxide and carboxymethylcellulose). *Clonostachys* strains in the mixtures of additives suppressed *Botrytis* sporulation on eucalyptus leaf discs between 15°C and 25°C. At temperatures from 15°C to 25°C, *Clonostachys* suppressed *Botrytis* sporulation on leaf discs when compared to control but, at 10°C, the pathogen sporulation inhibition was significantly lower when compared to that from 15°C to 25°C. The obtained results suggest that: 1- potassium and calcium carbonate, cornstarch, sodium alginate, titanium dioxide, and carboxymethylcellulose can be used in formulations containing *Clonostachys* spores; 2- *Clonostachys* can be used between 15°C and 25°C but, below this range, other strategies should be adopted.

Keywords: Biocontrol agent, inhibition of *Botrytis* conidial germination, formulation additives, *Eucalyptus globulus*

RESUMO

Musiet, D.; Sanfuentes, E.; Sossa, K.; Bettiol, W. *Clonostachys rosea*: efeito de aditivos na sua eficiência em inibir a esporulação de *Botrytis cinerea* em discos de folhas de eucaliptos. ***Summa Phytopathologica***, v.51, p.1-7, 2025.

Neste estudo foram avaliados: os efeitos de aditivos, bem como de suas misturas, usadas em formulações de pesticidas, sobre a germinação de conídios de *Clonostachys rosea;* e a eficiência de *C. rosea* isolados A10 e A11, com e sem mistura dos aditivos, na inibição da esporulação de conídios *Botrytis cinerea* em discos de folhas de *Eucalyptus globulus* em diferentes temperaturas. A germinação de conídios dos isolados de *Clonostachys* em carbonato de potássio e de cálcio (0,5, 1, 2 g L^{-1}) e amido de milho e alginato de sódio (10, 15, 20 g L^{-1}) foi similar ao controle com água (93,7% - 97,3%); e para o dióxido de titânio (2, 4, 6 g L^{-1}) e carboximetilcelulose (0,5, 1, 2 g L^{-1}) a inibição foi entre 7 e 16%. Glicerol inibiu completamente a germinação de conídios. A viabilidade dos conídios de *Clonostachys* foi mantida nas misturas contendo aditivos (carbonato de potássio e de cálcio, dióxido de titânio e carboximetilcelulose). Os isolados de *Clonostachys* nas misturas dos aditivos suprimiram a esporulação de *Botrytis* nos discos de folhas de eucaliptos entre 15 °C e 25 °C. Nas temperaturas entre 15 °C a 25 °C, *Clonostachys* suprimiu a esporulação de *Botrytis* nos discos de folhas quando comparado com o controle, mas a 10 °C a inibição da esporulação do patógeno foi significativamente menor do que quando comparado com as temperaturas entre 15 °C a 25 °C. Esses resultados sugerem que: 1-carbonato de potássio e de cálcio, amido de milho, alginato de sódio, dióxido de titânio e carboximetilcelulose podem ser utilizados em formulações contendo esporos de *Clonostachys*; 2-*Clonostachys* pode ser utilizado entre 15°C e 25°C, mas abaixo desta faixa outras estratégias devem ser usadas.

Palavras-chave: agente de biocontrole, inibição da germinação os conídios de *Botrytis,* aditivos de formulação, *Eucalyptus globulus*

Botrytis cinerea Pers. ex Fr. is the causal agent of gray mold in more than 200 crop species worldwide [45]. Gray mold is a serious disease affecting commercially important crops such as cucumbers (*Cucumis sativus* L.), grapes (*Vitis vinifera* L.), lettuces (*Lactuca sativa* L.), raspberries (*Rubus idaeus* L.), tomatoes (*Solanum lycopersicum* L.), strawberries (*Fragaria* x *ananassa* Duch.), and both ornamental and forestry plants [1, 14, 20, 46]. In *Eucalyptus* nurseries, gray mold can cause up to 50% plantlet losses [46] and unusually attack plantations, reducing plant growth within one year [1]. Control of gray mold in eucalyptus nurseries has been successful with fungicide sprays [1]; however, problems with resistant populations of *B. cinerea* have been reported for different crops [4, 13, 23, 39]. Suppression of *B. cinerea* sporulation on senescent tissues, reducing the pathogen inoculum, has been successfully adopted as a biological control strategy against gray mold [38, 12].

Biocontrol agents studied to control gray mold include *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams, which has shown the best results in suppressing *Botrytis* sporulation [10, 11, 12, 30, 38]. *Clonostachys rosea*, a saprophyte fungus found in crop residues [11, 36, 40], adopts competition for nutrients and substrate, as well as colonization of tissue wounds, as major mechanisms against *B. cinerea* [30, 37, 38]. Successful cases of *C. rosea* controlling *Botrytis* in strawberries, begonias (*Begonia elatior* Hort. ex Steud) and eucalypti (*Eucalyptus globulus* Labill.) were reported by Cota et al. [10, 11, 12], Fujinawa et al. [20], and Zaldúa and Sanfuentes [46]. In addition to controlling *Botrytis* and other plant pathogenic fungi [28, 36], *Clonostachys* has been reported parasitizing insects and nematodes [2, 26, 27, 28, 36], showing to be promising for the development of bioprotectors aimed at integrated disease management.

Easy inoculum production in grains (e.g., rice and oat), low risk of allergy to workers exposed to the spores, and low ecological risk [35, 37] are some of the advantages of using *Clonostachys* as bioagent for disease control. Even though these advantages are well documented, there are few *Clonostachys*-based products in the market, partially due to problems concerning the formulation of this bioagent. Considering the growing market for *C. rosea*, as well as the limitations related to its formulation, the objectives of the present study were to evaluate: 1- the effects of additives (titanium dioxide, carboxymethylcellulose, potassium and calcium carbonate, cornstarch, sodium alginate, and glycerol) and their combinations, used to develop biopesticide formulations, on the conidial germination of *C. rosea* strains, and 2- the efficacy of *Clonostachys* strains A10 and A11, alone or in mixture of additives, on the inhibition of *Botrytis cinerea* sporulation on *E. globulus* leaf discs at different temperatures.

MATERIALS AND METHODS

The assays were conducted at "Laboratorio de Patología Forestal", Universidad de Concepción, located in Concepción, Región del Bio Bio, Chile.

Clonostachys rosea and *Botrytis cinerea* strains and inoculum preparation

Clonostachys rosea strains A10 and A11, isolated from Carlos Douglas Nursery (Región del Bio Bio, Chile) and previously selected as antagonists of *B. cinerea* in *E. globulus,* were used in these studies [29, 46]. The isolates were deposited at "Laboratorio de Patología Forestal", Universidad de Concepción. For inoculum preparation, *C. rosea* strains were grown on Potato-Dextrose-Agar (PDA) in Petri plates at 25 ± 2°C and 12 h photoperiod for 15 days. The conidia were suspended in 0.04% Tween 80 solution with distilled water (0.01% v/v), and the concentration was adjusted to 1.0×10^6 conidia mL^{-1} using a Neubauer chamber. *Botrytis cinerea* isolate was obtained from symptomatic *E. globulus* seedlings from Carlos Douglas Nursery, multiplied as previously described for *C. rosea*, and the suspension was adjusted to 1.0×10^6 conidia mL^{-1}.

Effects of additives on conidial germination of *Clonostachys rosea*

One glass slide methodology was used to evaluate the effects of titanium dioxide (2, 4 and 6 g L^{-1}), carboxymethylcellulose (0.5, 1 and 2 g L^{-1}), potassium and calcium carbonates (0.5, 1 and 2 g L^{-1}), cornstarch (10, 15 and 20 g L^{-1}), sodium alginate (10, 15 and 20 g L^{-1}), and glycerol (20, 35 and 50 g L^{-1}; Table 1) on conidial germination of *C. rosea* strains A10 and A11. The conidial suspension (20 µL) of *C. rosea* strains (1×10^6 conidia mL^{-1}) was deposited onto one glass slide with water-agar 1% and the additives (previously autoclaved at 121°C for ٢٠ min; pH 6 before sterilization) at the concentrations described on Table 1. After incubation for 24 h (25 ± 2°C), in the dark, in a humid chamber, germination was interrupted by adding 10 µL lactophenol cotton blue dye to each droplet. Subsequently, 100 conidia were examined under a light microscope at 400 X (Zeiss). Conidia with germ tubes that were at least 1.5 times the length of their largest diameter were considered germinated. Trials were set up in a completely randomized design with three replicates, and each replicate consisted of one glass slide and two microscopic fields of view (two drops each glass slide). The values were represented as percentages of germinated conidia. According to the results obtained from an individual evaluation of additives, the mixtures containing a combination of titanium dioxide (4 g L^{-1}), carboxymethylcellulose (0.5 g L^{-1}), potassium and calcium carbonates (1 g L^{-1}), cornstarch and sodium alginate (15 g L^{-1}) (Table 1) were evaluated using the methodology described above. Glycerol was not included in the mixture because it completely inhibited conidial germination. Germination inhibition was calculated by the formula: Inhibition (%) = [(%Gc - %Gt) / %Gc] x 100; where %Gc is the percentage of conidial germination in the control, and %Gt is the percentage of conidial germination in the treatment.

Efficacy of *Clonostachys rosea* strains on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures

Experiments were conducted, as described by Peng & Sutton [30], to evaluate the effects of *C. rosea* on the inhibition of *B. cinerea* sporulation on eucalyptus leaf discs. One-centimeter-diameter leaf discs of young *E. globulus* leaves were surface disinfected in 70% ethanol (1 min) and in 0.5% sodium hypochlorite (1 min) and then rinsed three times with sterile distilled water. Leaf discs were dipped in conidial suspension of *C. rosea* strains A10 and A11 (1×10^7 conidia mL^{-1}) for one minute. After inoculation, 15 *E. globulus* leaf discs were placed in glass Petri dishes (150 x 20 cm) over humidified sterile absorbent paper (Whatman n. 1) with 5 mL sterile water. Following incubation of Petri dishes at 10, 15, 20, 25 or 30ºC for 24 h (12 h light), all discs were sprayed with 1 mL *B. cinerea* (1×10^5 conidia mL^{-1}). After inoculation, the discs were incubated for 24 h under the same previously described conditions. Discs were then transferred to paraquat chloramphenicol

agar (PCA) medium in Petri dishes [30] and incubated at the same temperatures described above for 10 days (Table 2). Paraquat was used in the medium because it desiccates the green parts of the plant, and chloramphenicol was employed to prevent bacterial growth. *Botrytis cinerea* sporulation was assessed based on a rating scale according to the percentage of disc area covered with fungal conidiophores, adapted by Peng and Sutton [30]: 1 = without *B. cinerea* sporulation; 2 = sporulation < 25% leaf disc area; 3 = sporulation between 25% and 50% leaf disc area; 4 = sporulation >50% leaf disc area; 5 = complete sporulation but few conidiophores over the leaf disc area; 6 = complete sporulation and conidiophores regularly distributed over the leaf disc area, and 7 = complete colonization and conidiophores homogeneously distributed throughout the leaf disc area. Trials were set up in a completely randomized design with four replicates, and each replicate consisted of one Petri dish and 15 *E. globulus* leaf discs.

Efficacy of *Clonostachys rosea* in mixture of additives on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures

The previously described methodology was also adopted to study the efficacy of *C. rosea* in two mixtures containing additives on the inhibition of *B. cinerea* sporulation on *E. globulus* leaf discs. Mixture 1 contained: titanium dioxide 4 g L^{-10}, potassium carbonate 1 g L^{-1}, cornstarch 10 g L^{-1}, and carboxymethylcellulose 0.5 g L^{-1}; the pH of the conidial suspension was between 5.6 – 5.8. Mixture 2 was similar to Mixture 1 but the pH of the conidial suspension was 3.5, obtained with lactic acid. The concentrations of *C. rosea* CR A10 and CR A11 were 1 x 10^7 conidia mL^{-1}. The effect of *Bacillus subtilis* QST713 (Serenade® WP - Bayer - 3.5 g L^{-1}) and iprodione (Iprodion 50WP – Agrospec - 2 g L^{-1}) on the control of *B. cinerea* sporulation on *E. globulus* leaf discs was also evaluated as a positive control. The treatments are shown in Table 3. Plates were incubated at 15, 20 and 25ºC. *Botrytis cinerea* sporulation was assessed using the rating scale mentioned above. Trials were set up in a completely randomized design with four replicates, and each replicate consisted of one Petri dish and 15 discs.

Statistical analysis

For each experiment, the dataset was first analyzed to assess whether the data were compliant with the assumptions for parametric analyses. Therefore, datasets were used to build histograms and density plots. Results were subject to Shapiro-Wilk's test for normality. Additionally, homogeneity of variance was analyzed using Barlett's and Levene's tests. Data compliant with normality and homogeneity of variance were analyzed through analysis of variance (ANOVA) and multiple comparisons through Tukey's HSD or Fisher's LSD tests. For datasets non-compliant with the assumptions of ANOVA, data were analyzed either through Generalized Linear Models (GLM) or through non-parametric analyses of variance. Both GLM with binomial distributions and logit link were used to estimate the effects of treatments on the spore germination rate for each isolate. P-value was calculated by likelihood ratio test with Chi-square distribution. Significant levels were adjusted with single-step method. Non-parametric analysis of variance was done through Kruskal-Wallis H test and multiple comparison between treatments were done through Dwass, Steel, Critchlow and Fligner's (DSCF) tests. All statistical analyses were conducted by using R 3.3.2 [32] and the packages "multcomp" [25], "car" [19] and "PMCMRplus" [31].

RESULTS

Effects of additives on conidial germination of *Clonostachys rosea*

According to the assays for assessing the effects of additives on conidial germination, *C. rosea* strains A10 and A11 showed 97.3% and 93.7% conidial germination in the control treatments, respectively, and behaved similarly regarding conidial germination for all additives, except glycerol. Potassium and calcium carbonates (0.5, 1 and 2 g L^{-1}), as well as cornstarch and sodium alginate (10, 15 and 20 g L^{-1}), practically did not affect the conidial germination of *C. rosea* strains A10 and A11, showing germination percentages similar to those of the control. Titanium dioxide (2, 4, 6 g L^{-1}) and carboxymethylcellulose (0.5, 1, and 2 g L^{-1}) inhibited between 7% and 16% conidial germination of strains A10 and A11. Glycerol completely inhibited conidial germination at the concentrations 20, 35 and 50 g L^{-1} for both strains (Table 1).

Conidial germinations of *C. rosea* strains A10 and A11 were higher than 91% in the presence of both the mixture containing sodium alginate (15 g L^{-1}), potassium and calcium carbonates (1 g L^{-1}), carboxymethylcellulose (0.5 g L^{-1}) and titanium dioxide (4 g L^{-1}), and the mixture containing cornstarch (15 g L^{-1}), potassium and calcium carbonates (1 g L^{-1}), carboxymethylcellulose (0.5 g L^{-1}) and titanium dioxide (4 g L^{-1}). Therefore, the concentrations 15 g L^{-1} sodium alginate and cornstarch, 1 g L^{-1} potassium and calcium carbonate, 0.5 g L^{-1} carboxymethylcellulose and 4 g L^{-1} titanium dioxide were selected to prepare eight mixtures using these additives. The mixtures of these products did not inhibit the conidial germination of *C. rosea* strains (Table 1).

Efficacy of *Clonostachys rosea* strains on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures

At 30°C, there was almost no *B. cinerea* sporulation on *E. globulus* leaf discs and very little sporulation in the control. At 15, 20 and 25°C, the two *Clonostachys* strains suppressed *B. cinerea* sporulation on *E. globulus* leaf discs, reducing the pathogen sporulation rate from 4.5 to 1.2; 5.0 to 1.0, and 3.4 to 1.0, considering the scale of Peng and Sutton [30], when leaf discs were incubated at 15, 20 and 25°C, respectively, while a significant pathogen sporulation could be observed in the control. At 10°C, pathogen sporulation was significantly lower when compared to that at 15, 20 and 25°C (Table 2). *Clonostachys rosea* A10 was less effective than A11 in suppressing *B. cinerea* sporulation at 15°C.

Efficacy of *Clonostachys rosea* in mixture of additives on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures

At 25°C, both *C. rosea* strains (A10 and A11), applied alone and in mixture with the additives, reduced *Botrytis* sporulation on *E. globulus* leaf discs, similarly to the fungicide iprodione. At 20°C, *C. rosea* strains A10 and A11 were more efficient than the fungicide, while at 15°C the fungicide was more efficient than *C. rosea* strains in reducing *Botrytis* sporulation on *E. globulus* leaf discs (Table 3). The two *Clonostachys* strains, in mixture of titanium dioxide 4 g L^{-1}, potassium carbonate 1 g L^{-1}, cornstarch 10 g L^{-1}, and carboxymethylcellulose 0.5 g L^{-1}, at both pH (5.6 and 3.5), similarly suppressed *Botrytis* sporulation on *E. globulus* leaf discs at 15, 20 and 25°C (Table 3). *Clonostachys rosea*

Table 1. Effects of additives on the conidial germination of *Clonostachys rosea* strains A10 and A11.

Treatment	Concentration (g L^{-1})	Conidial germination (and inhibition) (%)	
		Strain A10	Strain A11
Carboxymethylcellulose (CMC)	0.5	82.3 (15) bcde	81.3 (13) ef
	1	86.3 (11) bcd	79.0 (16) ef
	2	83.3 (14) bcde	81.3 (13) ef
Cornstarch (CS)	10	96.0 (1) ab	94.0 abc
	15	94.0 (3) ab	92.3 (1) abcd
	20	93.0 (4) abc	92.3 (1) abcd
Sodium alginate (SA)	10	89.0 (8) abc	94.0 abc
	15	95.0 (2) ab	94.0 abc
	20	94.0 (3) ab	93.0 (1) abc
Potassium carbonate (PC)	0.5	99.0 a	96.7 ab
	1	97.7 ab	98.3 a
	2	99.0 a	98.7 a
Calcium carbonate (CC)	0.5	91.0 (6) bc	93.0 (1) abc
	1	92.0 (5) abc	88.3 (6) cdef
	2	93.3 (4) ab	90.0 (4) bcd
Titanium oxide (TO)	2	86.3 (11) bcd	87.0 (7) def
	4	85.7 (12) bcd	85.0 (9) ef
	6	82.7 (15) bcde	84.3 (10) ef
Glycerol (G)	20	5.1 f	0.0 g
	35	2.5 f	0.0 g
	50	0.0 f	0.0 g
Control (water)		97.3 ab	93.7 abc
Mixture of additives			
1 - TO +SA + PC	(4 + 15 + 1)	92.7^{ns}	93.6^{ns}
2 - TO + SA + PC + CMC	(4 + 15 + 1 + 0.5)	94.3	94.6
3 – TO + SA + CC	(4 + 15 + 1)	91.3	94.0
4 – TO +SA + CC + CMC	(4 + 15 + 1 + 0.5)	91.7	93.3
5 – TO + CS + PC	(4 + 15 + 1)	92.0	95.3
6 – TO + CS + PC + CMC	(4 + 15 + 1 + 0.5)	94.0	93.0
7 – TO + CS + CC	(4 + 15 + 1)	94.3	93.7
8 – TO + CS + CC + CMC	(4 + 15 + 1 + 0.5)	93.3	93.0
9 - Control (water)	-	93.0	92.7

Mean values followed by the same letters for each strain are not significantly different according to Tukey's test ($p < 0.05$). Values in parentheses indicate the % inhibition of conidial germination. ^{ns} = not significant.

Table 2. Efficacy of *Clonostachys rosea* strains A10 and A11 on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures based on Peng and Sutton [30] scale.

Treatment	10 ºC	15 ºC	20 ºC	25 ºC	30 ºC
Control without pathogen	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a
Control with pathogen	2.4 c	4.5 c	5.0 b	3.4 b	1.2 a
C. rosea A10	1.7 b	1.4 b	1.1 a	1.0 a	1.0 a
C. rosea A11	1.6 b	1.2 a	1.0 a	1.0 a	1.0 a

Mean values followed by the same letters on the line are not significantly different according to Kruskal-Wallis test ($p < 0.05$). *C. rosea* A10 and A11 conidial suspension (1 x 10^7 conidia mL^{-1}) in water. Sporulation rate scale: 1 = without *B. cinerea* sporulation, 2 = sporulation <25% leaf disc area, 3 = sporulation between 25% and 50% leaf disc area, 4 = sporulation >50% leaf disc area, 5 = complete sporulation but few conidiophores over the leaf disc area, 6 = complete sporulation and conidiophores regularly distributed over the leaf disc area, and 7 = complete colonization and conidiophores homogeneously distributed throughout the leaf disc area [30].

Table 3. Efficacy of conidia of *Clonostachys rosea* strains A10 and A11 in mixture with additives (titanium dioxide, potassium carbonate, cornstarch and carboxymethylcellulose) (Mixture 1 and 2) on the inhibition of *Botrytis cinerea* sporulation on *Eucalyptus globulus* leaf discs at different temperatures**.**

Treatment/Temperature	Leaf disc colonization rating average					
	15 °C		20 °C		25 °C	
Control without pathogen	1.00 ± 0.00	e	1.00 ± 0.00	E	1.00 ± 0.00	c
Control with pathogen	3.67 ± 0.79	a	4.42 ± 0.56	A	2.87 ± 0.88	a
C. rosea A10	1.80 ± 0.57	b	1.25 ± 0.43	D	1.00 ± 0.00	c
C. rosea A11	1.62 ± 0.63	bc	1.32 ± 0.50	D	1.00 ± 0.00	c
Mixture 1 - *C. rosea* A10	1.97 ± 0.63	b	1.38 ± 0.49	cd	1.00 ± 0.00	c
Mixture 1 - *C. rosea* A11	1.45 ± 0.64	c	1.30 ± 0.49	d	1.00 ± 0.00	c
Mixture 2** (pH 3.5) - *C. rosea* A10	1.70 ± 0.61	b	1.30 ± 0.49	d	1.02 ± 0.13	c
Mixture 2 (pH 3.5) - *C. rosea* A11	1.40 ± 0.49	cd	1.33 ± 0.51	d	1.02 ± 0.13	c
Bacillus subtilis QST713	3.28 ± 0.66	a	3.90 ± 0.81	b	2.28 ± 0.73	b
Fungicide (Iprodione)	1.30 ± 0.74	d	1.73 ± 0.73	c	1.03 ± 0.18	c

Mean values followed by the same letters in the column are not significantly different according to Kruskal-Wallis test ($p < 0.05$). Mixture 1 = titanium dioxide 4 g L^{-1}, potassium carbonate 1 g L^{-1}, cornstarch 10 g L^{-1}; carboxymethylcellulose 0.5 g L^{-1}. **Mixture 2 = Mixture 1 with pH of conidial suspension 3.5 obtained with lactic acid. *C. rosea* A10 and A11 conidial suspension (1 x 10^7 conidia mL^{-1}) in water. *C. rosea* CR A10 and CR A11: 1x10^7 conidia mL^{-1}. *Bacillus subtilis* QST713 (Serenade® WP - Bayer - 3.5 g L^{-1}). Fungicide (Iprodione) = (Iprodion 50WP – Agrospec - 2 g L^{-1}). Sporulation rate scale: 1 = without *B. cinerea* sporulation, 2 = sporulation <25% leaf disc area, 3 = sporulation between 25% and 50% leaf disc area, 4 = sporulation >50% leaf disc area, 5 = complete sporulation but few conidiophores over the leaf disc area, 6 = complete sporulation and conidiophores regularly distributed over the leaf disc area, and 7 = complete colonization and conidiophores homogeneously distributed throughout the leaf disc area [30].

strains A10 and A11 showed similar efficacy in inhibiting the pathogen sporulation, when compared to the fungicide, at 25°C; however, at 20°C, the strains were more efficient. Iprodione (Iprodion 50WP) inhibited *B. cinerea* sporulation on *E. globulus* leaf discs at all temperatures. *Bacillus subtilis* QST-713 (biofungicide Serenade®) did not reduce *Botrytis* sporulation on *E. globulus* leaf discs at 15°C but led to a small reduction at 20 and 25°C (Table 3).

DISCUSSION

Formulations play a fundamental role in maintaining the quality of bioagents by acting in the stabilization of microbial propagules, by protecting against adverse environmental conditions such as UV radiation and dry conditions, and by giving the antagonist a competitive advantage over plant pathogens and other native microflora agents [24, 42]. Formulation components are composed of three parts: active ingredient, carrier materials and additives [6, 7, 44]. Efforts have been dedicated to the production, formulation and application of bioagents, which are essential for obtaining effective biopesticides [41, 34]. No information has been found about the effects of formulation components on *Clonostachys* conidial germination, and such limited information about formulations on biological control products is due to the high interest of companies for patents.

Conidial germination of *C. rosea* strains (A10 and A11) for potassium and calcium carbonates, carboxymethylcellulose (0.5 − 2 g L^{-1}), cornstarch, sodium alginate (10 − 20 g L^{-1}), and titanium dioxide (2 − 6 g L^{-1}), individually and in mixtures containing combinations of 15 g L^{-1} sodium alginate and cornstarch, 1 g L^{-1} potassium and calcium carbonate, 0.5 g L^{-1} carboxymethylcellulose, and 4 g L^{-1} titanium dioxide, was similar to that of the control in water (Table 1). The efficacy of conidia of *C. rosea* strains (A10 and A11) in mixture with titanium dioxide, potassium carbonate, cornstarch and carboxymethylcellulose, and some mixtures are shown in Table 3. The present results indicate that the studied materials can be used for *C. rosea* formulation (Tables 1 and 3). This piece of information is important since each additive can provide several advantages for the preparation of *Clonostachys*-based formulations.

The addition of sunscreens is of great importance in the preparation of formulations because the structures of biocontrol agents are sensitive to the ultraviolet radiation (UV) [8, 9, 15, 17, 21]. Titanium dioxide, which is a sunscreen used in bioagent formulations [15, 22, 34, 44], reduced conidial germination by only 9% − 12% for both strains at 4 g L^{-1}, showing its compatibility with *Clonostachys* strains. However, when it was combined with other additives (Table 1), the inhibition rate was lower. Considering the importance of titanium dioxide in preventing conidial inactivation or death when applied under field conditions, the concentration 4 g L^{-1} can be considered.

Carboxymethylcellulose, cornstarch and sodium alginate are used in several formulation processes [3, 6, 16, 18, 34, 41, 43] and are also compatible with *Clonostachys* strains (Table 1). The viability of *Clonostachys* conidia was maintained in all mixtures containing carboxymethylcellulose, cornstarch, sodium alginate, titanium dioxide, potassium and calcium carbonate (Table 1). The results shown in Table 1 indicate the possibility of using these additives to prepare formulations with *Clonostachys* conidia. However, glycerol completely inhibited conidial germination at the concentrations 20, 35 and 50 g L^{-1}, for both A10 and A11, and was considered not compatible with such strains.

Clonostachys, as well as other antagonists, are temperature-dependent for pathogen growth and control [10, 38]. In the current study, the two *Clonostachys* strains (A10 and A11), alone or in mixture of additives, suppressed *Botrytis* sporulation on *E. globulus* leaf discs at 15, 20 and 25°C (Tables 2 and 3). The behavior of *Clonostachys* strains A10 and A11 was similar to that of the four *Clonostachys* strains studied by Cota et al. [10], which controlled *Botrytis* sporulation between 15°C and 25°C, peaking at around 25°C.

At 30°C, almost no *B. cinerea* sporulation on *E. globulus* leaf discs was observed for the control treatment; at 10°C, *Botrytis* sporulation was reduced (Table 2), also demonstrating its dependence on a suitable temperature for sporulating. These results show that both the bioagent and the pathogen depend on the temperature to act. The present results also suggest that *Clonostachys* strains have the best efficiency within the temperature range between 15°C and 25°C.

Since the studied *Clonostachys* strains did not show any action at 10°C, the use of chemical fungicide is suggested to control *B. cinerea* under such environmental conditions, which are common at nurseries in southern Chile during autumn.

One of the major problems in managing diseases caused by *Botrytis* is the emergence of populations resistant to one or more fungicides [5, 14, 33]. Therefore, considering the efficacy of *Clonostachys* in inhibiting *Botrytis* sporulation, the use of this biological control agent is suggested for *Eucalyptus* integrated management program, since it may also reduce problems with the emergence of isolates resistant to the major fungicides used in the crop.

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