In vitro effect of four biofungicides on control of *Ciborinia camelliae* Kohn

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Introduction

One of the major diseases affecting camellia is camellia flower blight, which is caused by *Ciborinia camelliae* Kohn, a quarantine pathogen included in the EPPO A2 List (EPPO, 1997). In Europe, this ascomycete was first detected in 1998 (Lane & Beales, 2001) and now is widespread in France, Spain, Italy, United Kingdom and Switzerland (EPPO, 2009), affecting species, hybrids and cultivars of the genus *Camellia*.

There is not any control method completely efficient against this pathogen. Those previously described either have not been totally effective, or are arduous and difficult to apply by growers, or require the systematic use of chemical fungicides (Van Toor *et al.*, 2005). For instance, attempts were made to control *Ciborinia camelliae* using chemical fungicides on the flowers to avoid ascospore infection, but having limited success (Taylor & Long, 2000). Only removal and burning of fallen flowers that could contain sclerotia can be efficient, but it is an extremely tedious method.

In the last years several biopesticides have been developed as an alternative to the use of chemicals for pest and disease control. These biological products are specific, environmental friendly and tend to reduce the development of pathogen resistance (Boyectchko *et al.*, 1998).

The aim of the present work was to assess the *in vitro* effect of four biofungicides on mycelial growth and viability of *Ciborinia camelliae* sclerotia.

Materials and Methods

A *Ciborinia camelliae* isolate was obtained from symptomatic flowers of *Camellia japonica* plants belonging to the Camellia Collection of the Estación Fitopatolóxica do Areeiro (Pontevedra, NW Spain). Samples of mycelia ring around the calyx were taken and cut and surface disinfected following the method proposed by Van Toor *et al.* (2000).

Disinfected ring fragments were placed onto Petri dishes with PDA (potato-dextrose-agar) culture media and incubated at 18 ºC until *Ciborinia camelliae* mycelial growth and sclerotia formation were observed (Figure 1).

Figure 1. *Ciborinia camelliae* mycelium and sclerotia in PDA medium

Pure cultures and sclerotia of the isolate were obtained and identified by morphological features in culture media and by molecular analysis of DNA of the ITS region (White *et al.*, 1990) and beta-tubulin gene (Glass & Donaldson, 1995). To study the effects on mycelial growth and sclerotia formation of *Ciborinia camelliae*, four biological products containing antagonist or

parasites of plant fungi were used: TIFI (*Trichoderma atroviride* strain 898G, 2 x 10^8 CFU/g, 2% w/w) (Giten Biological, Tarragona, Spain), CONTANS® WG (*Coniothyrium minitans* strain CON/M/91-08, 1 x10^{12} CFU/kg, 5% w/w) (Belchim Crop Protection, Valencia, Spain), KONI WG (*Coniothyrium minitans* strain K1, 5 x10^7 CFU/g, 5% w/w) (Futureco, Barcelona, Spain), and ACTINOVATE® WP (*Streptomyces lydicus* strain WYEC 108, 3,5 x10^8 CFU/g, 0,0371% w/w) (Futureco, Barcelona, Spain).

Trichoderma species, frequently found in the soil, have been widely studied and proved to be efficient on the protection of several plant species, or to induce resistance, against *Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria solani*, *Phytophthora capsici* or *Pseudomonas syringae*, among others (Harman *et al.*, 2004), thus many commercial biopesticides contain *Trichoderma* species as active ingredient (Vinale *et al.*, 2008). Its effect on the control of camellia flower blight has been scarcely studied. Van Toor *et al.* (2005) found that the application in the field of different strains of *Trichoderma virens* and *T. viride* isolated from decaying *Cborinia camelliae* sclerotia could reduce *in vitro* the number of viable sclerotia in 53% and 43%, respectively.

Coniothyrium minitans, recently reclassified as *Paraconiothyrium minitans* (Verkley *et al.*, 2004), can infect sclerotia of many Ascomycota, among them several species of *Sclerotinia* (Whipps *et al.*, 2008), even though the strains tested so far were no effective on the control of *Ciborinia camelliae* (McLean *et al.*, 2004; Van Toor *et al.*, 2005).

Streptomyces lydicus, an actinomycete that produces a large amount of antifungal metabolites and extracellular chitinases, has shown *in vitro* effectiveness for the control of several plant pathogens, such as *Pythium ultimum* (Mahadevan & Crawford, 1997). However, its use as a biocontrol agent of camellia flower blight has not been studied yet.

An *in vitro* assay was designed to study the effect of the four biofungicides on the mycelial growth of *Ciborinia camelliae*. For each biofungicide, stock solutions (100 mg active ingredient (AI) l^{-1}) were prepared in sterile distilled water. Individual agar plugs (7 mm in diameter) were taken from the margin of an actively growing culture of the *Ciborinia camelliae* isolate and placed in the center of 9 cm plastic Petri dishes (one agar plug per dish) containing PDA amended with aliquots of each stock solution to provide concentrations of 0.01, 0.1, 1 and 10 mg AI l^{-1}. Fungicides were added to the PDA medium after autoclaving, when the agar had cooled. Petri dishes containing only PDA and sterile distilled water were used as control. A parallel assay was performed to study the effect of the same concentrations of the four biofungicides on the viability of *Ciborinia camelliae* sclerotia. Sclerotia were obtained from *in vitro* cultures of the *C. camelliae* isolate and were cleaned free of agar and placed in the center of Petri dishes (one sclerotia per dish).

In both assays, there were five replicates for each product and concentration, and the control. Experiments were conducted in triplicate.

Plates were sealed with Parafilm® and incubated at 20 ºC in darkness. The radial growth of mycelia was measured every day in two perpendicular directions on each cultured plate. Measurements were averaged, and the diameter of the inoculation plug substracted. Percent growth reduction was determined at each concentration of fungicide tested, compared with growth on non amended medium. Data were subjected to a one way analysis of variance (ANOVA). Comparison of means was performed using the Duncan test for $p \leq 0.05$.

In all experiments, agar plugs and sclerotia which did not yield growth in fungicide-amended plates of any treatment were transferred to freshly prepared plates of PDA medium without fungicides, in order to determine whether the activity of the corresponding active ingredient was fungistatic or fungicidal.

Results

The four formulations studied both inhibited mycelial growth and sclerotia viability of *Ciborinia camelliae*. The highest concentrations, namely 1 and 10 mg AI l^{-1}, of *Streptomyces lydicus* and *Conyothirium minitans* products completely inhibited mycelial growth (Table 1) (Figure 2). At 0.1 mg AI l^{-1}, *Conyothirium minitans*, formulated either as Contans or Koni, resulted in more than 90% inhibition. All biofungicides but *Streptomyces lydicus* showed a high efficacy to inhibit *Ciborinia camelliae* mycelial growth even at the lowest dose, 0.01 mg AI l^{-1}, with more than 80% inhibition.

Effects of the four biofungicides on sclerotia viability were similar to those on mycelial inhibition. Active ingredients showing the best results were *Trichoderma atroviride* and *Conyothirium minitans*, which inhibited development of *Ciborinia camelliae* sclerotia at all doses (Table 2). *Streptomyces lydicus* could inhibit sclerotia development only at 10 mg AI l^{-1}, the highest dose tested, whereas, at lower concentrations, sclerotia germinated producing mycelium.

For all biofungicides, the activity of the corresponding active ingredient was fungicidal, both hindering mycelial growth and sclerotia development.

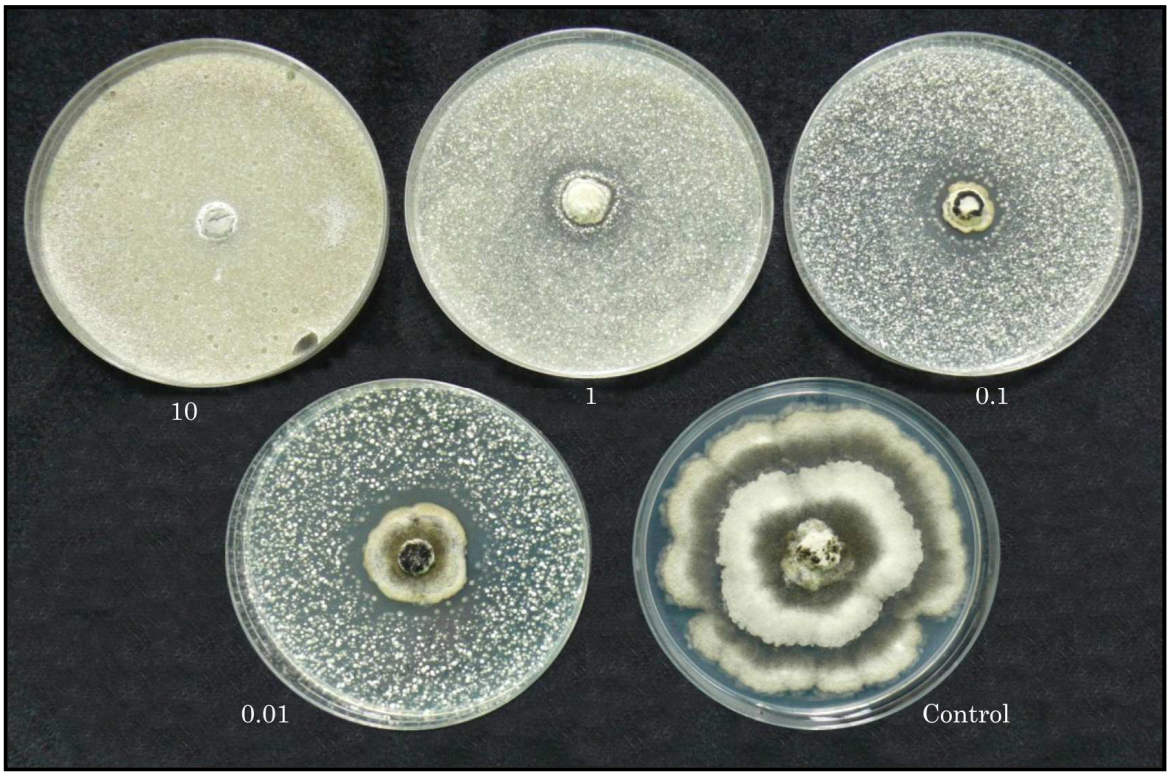

Figure 2. Mycelial growth of *Ciborinia camelliae* at control, 0.01, 0.1, 1 and 10 mg AI l^{-1} of a *Streptomyces lydicus* product.

Discussion

Mycelial growth and viability of sclerotia of *Ciborinia camelliae* were inhibited *in vitro* by all biofungicides tested, but the pathogen showed higher sensitivity to those including *Trichoderma atroviride* or *Conyothirium minitans* as active ingredient. Best inhibitory results were achieved with products containing *Coniothyrium minitans*, which resulted in mycelial growth inhibition higher than 90% at a dose of only 0.1 mg AI l^{-1} and hindered mycelial growth from sclerotia at all assayed doses, suggesting their potential efficacy to control camellia flower blight.

These biofungicides could be used both to prevent and control the disease caused by *Ciborinia camelliae*, although further research is needed to study minimum effective doses, their sensitivity to environmental factors and the convenience of annual dressings in affected plants, in order to achieve a progressive reduction of the pathogen inoculum potential in soil and to avoid development of fungal resistance. All these factors would allow to register these biofungicides as elective active ingredients in the treatment of camellia flower blight caused by *Ciborinia camelliae.*

Table 1. Effect of different doses of *Streptomyces lydicus, Trichoderma atroviride* and *Coniothyrium minitans* products on mycelial growth inhibition (%) of *Ciborinia camelliae*.

	Mycelial growth inhibition (%)			
Doses (mg AI l^{-1})	**Biofungicides**			
	S. lydicus	***T. atroviride***	***C. minitans* (Contans)**	***C. minitans* (Koni)**
0.01	47.4 a	82.0 a	89.1 a	80. 4 a
0.1	84.9 b	84.0 ab	98.1 b	91.5 b
1	97.1 c	85.2 ab	100 b	100 c
10	100 c	87.3 b	100 b	100 c

In each column, values with the same letter are not significantly different for $p \leq 0.05$.

Table 2. Effect of different doses of *Streptomyces lydicus, Trichoderma atroviride* and *Coniothyrium minitans* products on viability of *Ciborinia camelliae* sclerotia.

Viability of *Ciborinia camelliae* sclerotia				
Doses (mg AI l^{-1})	**Biofungicides**			
	S. lydicus	***T. atroviride***	***C. minitans* (Contans)**	***C. minitans* (Koni)**
Control	+	+	+	+
0.01	+	-	-	-
0.1	+	-	-	-
1	+	-	-	-
10	-	-	-	-

+: viability of sclerotia; -: no viability of sclerotia

Acknowledgements

Authors thank Ms. Carmela Menduiña Santomé, laboratory technician of the Estación Fitopatolóxica do Areeiro, for her skillful technical assistance. We also wish to thank companies Belchim Crop Protection (Valencia, Spain), Futureco (Barcelona, Spain) and Giten Biological (Tarragona, Spain) for supplying the biofungicides used in the present work.

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