

DEVELOPMENT OF MUTANTS OF *CONIOTHYRIUM MINITANS* WITH IMPROVED EFFICIENCY FOR CONTROL OF *SCLEROTINIA SCLEROTIORUM*

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Abstract: *Coniothyrium minitans* (CM) is hyperparasitic to *Sclerotinia sclerotiorum* (SS), a pathogen of many economically important crops. In this paper, we describe the isolation of improved mutants of CM, using a UV – irradiation regime, with altered chitinase production and tolerance to high concentration of iprodione, which are effective against SS. Three out of the 59 mutants obtained inhibited the mycelial growth of CM. Infectivity of sclerotia by the new mutants was assayed by the plant-tissue-based system using carrot segments. More than 80% of sclerotia were colonized by the mutants and the wild-type CM. The mutant strains retained ability to produce significant amounts of chitinase. The mutants differed from their wild-type strain in appearance, morphology and sporulation. In conclusion, the results presented here provide evidence that the new biotypes of *C. minitans* are effective in controlling *S. sclerotiorum*.

Key words: *Coniothyrium minitans*, *Sclerotinia sclerotiorum*, iprodione, resistance, biological control, antibiotic production

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogen that causes serious losses in many agricultural crops both in storage and in the field. The pathogen forms sclerotia which are resistant to drought, heat and fungicides and may remain viable in the soil for many years (Coley-Smith 1979).

As environmental concern over the use of agrochemicals increases, fewer pesticide products are now available to the grower. There is a need for additional methods of control of *Sclerotinia* spp., especially for *S. sclerotiorum* in field crops. In the field, control of *Sclerotinia* species has been obtained by use of soil drenches of dicarboximide fungicides such as iprodione or vinclozolin, but recently this procedure has begun to fail because of enhanced degradation of the chemicals. The levels of control obtained with others fungicides, including benzimidazole compounds, are inadequate. Furthermore, the development of tolerance by plant pathogens to benzimidazole compounds (Dekker and Georgopoulos 1982) and reports of mutagenicity of such compounds (Davidse and Flach 1977) justifies the need for more adequate and safer method of disease control.

The mechanisms by which antagonistic microorganisms are believed to control plant diseases are generally categorized as competition, parasitism, and antibiosis. The exact roles of antibiotics and enzymes in biological

control in the natural environment have been more difficult to establish. Some of these compounds are involved in plasmolysis of hyphal tip cells or inhibition of hyphal growth of some pathogens. They may also protect seedlings from decay. However, the support for this type of activity has come mainly from the identification of metabolites found in culture that inhibit pathogens *in vitro* (Graeme-Cook and Faull 1991; Wilhite *et al.* 1994). Unfortunately, most of the potentially promising biological control agents may reproduce only asexually which limits our ability to improve on wild type activity. Fungi without functioning sexual stages may be altered genetically by conventional mutation techniques. For example, the efficiency of a fungus in biological control might be enhanced by modification of genes promoting antibiotic and enzyme production or resistance to fungicides (Graeme-Cook and Faull 1991). Furthermore, potential fungal antagonists may not tolerate fungicides, such as the benzimidazoles and dicarboximides that are routinely used as agents of chemical control. UV mutagenesis can also be used to induce improved resistance to fungicides. The use of fungicide resistant mutants could increase the potential for integrated control and provide an agent that could be “target” (Baker 1983, 1989).

As the major survival structures formed by this pathogen are sclerotia, considerable interest has been focused on the selection of sclerotial mycoparasites as a source of

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biocontrol agents. A wide range of fungi have been recorded and *C. minitans* is included in this group.

Coniothyrium minitans Campbell is a specific sclerotial mycoparasite and has been found in sclerotia of *Sclerotinia* and *Sclerotium* species. *C. minitans* is able to survive and spread efficiently, thus providing a rapid biocontrol action against *sclerotinia* disease (Budge and Whipps 1991; Huang and Kozub 1991; Whipps *et al.* 1993).

This biocontrol agent shows the ability to control sclerotia by growing mycelium which invades sclerotia intercellularly and intracellularly (Jones 1970).

The capability of penetrating the melanized rind wall was suggested to be an effect of the hyphal pressure as well as the disintegration of host cell walls by enzymatic action (Huang and Hoes 1976; Jones *et al.* 1974). A major component of cell walls of *S. sclerotiorum* are β (1–3) glucan and chitin; lysis is caused by endo- and exo-glucanases (β (1–3) glucanase and chitinases) that are produced by *C. minitans*.

The objective of this paper was to isolate improved mutants of *C. minitans* B21 produced by ultraviolet light irradiation for tolerance to high concentration of iprodione and with altered antibiotic production, and to assess their ability as biocontrol agents of *S. sclerotiorum*.

MATERIALS AND METHODS

Microorganisms

C. minitans strain B21, was isolated from infected sclerotia of *S. sclerotiorum*, collected from bean residues. Sclerotia of *S. sclerotiorum* was isolated from infected tomato plants, originating from a naturally infested area in north of São Paulo State, Brazil.

Isolation of Mutants of *C. minitans*

Mutants were induced by ultraviolet light irradiation of conidia to a survival rate of 5%. The dose was 97.00 $\mu\text{w}/\text{cm}^2 \times 10$, at a distance of 17 cm. After irradiation, the conidia were plated directly into PDA medium supplemented with 100 $\mu\text{g}/\text{ml}$ of freshly prepared aqueous solutions of iprodione (Rovral 50 WP). All colonies resistant to iprodione were transferred to slope culture of PDA plus iprodione. The stability of resistance was examined by plating the colonies periodically on PDA with and without the fungicide.

Extraction of antibiotics in liquid culture

PDA medium was prepared and distributed into 250 ml sterile flasks (100 ml per flask). Three 5 mm mycelial discs from 12-day-old culture of the mutants of *C. minitans* were added to each flask. Five replicate flasks were prepared. The flasks were incubated on a rotatory shaker at room temperature for 12 days. The mycelium was filtered under vacuum using filter paper (Whatman n° 4) and the culture filtrate refiltered through a Millipore filter (0.22 μm) directly into sterile flasks. Culture filtrate from replicates flasks of each mutant was pooled together and each one was extracted with ethyl acetate by shaking with the aqueous fraction in a separating funnel. The heavier aqueous layers were allowed to separate out and run off

from the ethyl acetate. The latter were dried in a rotatory vacuum evaporator at 40°C. Once dried, the extract was re-dissolved in a minimal amount of the organic solvent.

The metabolites were tested against the pathogen by transferring 0.5 mm diameter mycelium discs of the pathogen onto three equidistant sites, 1 cm away from the edge of the PDA plates. Sterile filter paper discs (0.5 cm diameter) were soaked into metabolite extracts for 2 h and placed at the center of these plates. Filter paper discs soaked on ethyl acetate were used as control. The plates were incubated at 22°C for seven days. Biological activity of metabolite extracts were recorded as radius of mycelium growth towards *S. sclerotiorum*. Five replicate plates of each filtrate were made.

Parasitism of sclerotia

Sclerotia of *S. sclerotiorum*, produced in PDA medium culture, were dried, surface sterilized in 10% sodium hypochlorite for 2 minutes, rinsed in sterile distilled water, and soaked in a spores suspension of mutants of *C. minitans* (10^6 spores/ml). Those sclerotia were placed on moist sterilized vermiculite in a Petri dishes. Plates were sealed with parafilm to prevent desiccation and incubated at 22°C. After two weeks, sclerotia were removed, surface disinfected in 10% sodium hypochlorite for 2 min, rinsed in sterile water, and placed into fresh sliced carrots which had been surface sterilized for two minutes in sodium hypochlorite (10%). The Petri dishes were sealed and kept for two weeks at 22°C. The sclerotia were considered infected by the mutants when the pathogen was not able to grow on sliced carrots and showed mutants growth on the sclerotia. Five replications were used in each treatment, with ten sclerotia in each treatment.

For scanning electron microscopy (SEM), parasitized sclerotia were fixed in 2% glutaraldehyde, buffer-rinsed and post-fixed in 2% osmium tetroxide at 0°C for 2 h. The specimens were rinsed, and dehydrated in an ethanol series. Then, the material was critical-point-dried, mounted on SEM stubs, sputter-coated with gold and examined on an ultra-high performance SEM (Leo 982-Leica and Zeiss, Germany), operated at low KV.

Effect of temperature on mycelial growth and sporulation of mutants of *C. minitans*

Petri dishes containing PDA medium were inoculated centrally with 5 mm mycelial discs of mutants of *C. minitans* taken from the growing edge of 12-days-old PDA cultures incubated at 18, 22 and 28°C. Radial growth and sporulation were assessed 12 days after inoculation. Each treatment had three replicates. Measurement of the conidia of mutants was carried out as described in Tanaka *et al.* (1979). Fifty conidia were used per mutant.

Production of Chitinase

Erlenmeyer flasks (100 ml) containing 40 ml of Czapek medium plus 1% chitin were inoculated with 1×10^7 conidia ml. Flasks were shaken at 180 rev/min at 28°C for 5 days. Samples were centrifuged and the supernatants analyzed for the chitinolytic activity. The analysis was demonstrated in solutions assays using chromogenic chitin derivation as a substrate. Chitinase activity in extracts

was colorimetrically assayed using a carboxymethylchitin-remazol brilliant violet solution as substrate (CM-Chitin-RBV 2 mg/ml, Loewe Biochemica GmbH, Otterfing Bei München, Germany) (Wirth and Walf 1990).

RESULTS AND DISCUSSION

Fifty nine mutants of *C. minitans* B21, which tolerated 100 µg a.s. of iprodione, were isolated. They were tested for genetic stability and resistance to iprodione by letting them regrow on PDA medium supplied with iprodione and for their antagonism towards *S. sclerotiorum* (data not shown). Only 5 mutants were selected for the screening on the basis of increases on antibiotics production and mycoparasitism of sclerotia of *S. sclerotiorum*. All colonies (mutants) showing sectors, due to genetic instability, were discarded.

The growth of *S. sclerotiorum* was inhibited by metabolite extracts of five mutants of *C. minitans* B21: CM4b, CM 4c, CM7, CM10 and CM23 (Table 1). The test on PDA medium showed significant effects of the non-volatile antibiotic produced by mutants on the growth rates and morphology of the pathogen. The higher antibiotic activity observed for the mutants was probably due to new compounds not normally synthesized by the parental strain under such conditions. Those three mutants that exhibited better mycelial growth control also showed the best results on parasitism of sclerotia test. Examination of thin-layer chromatograms of these strains showed that several different compounds were being produced, and some of them were not present on wild-type (not shown here). Studies carried out *in vitro*, which correlate the presence of metabolite with the biological activity towards a pathogen, may not necessarily predict the *in vivo* activity of a metabolite (Fravel 1988).

Table 1. Parasitism of sclerotia of *S. sclerotiorum* by mutants of *C. minitans* B21, on freshly sliced carrots, *in vitro*, and effects of metabolic extracts produced by mutants on the growth of the pathogen

Mutants	Non-viable sclerotia [%]	*Inhibition of mycelial growth [%]
CMB21	100.00 a	61.50
CM4b	100.00 a	68.37
CM4c	100.00 a	62.75
CM23	80.00 ab	67.75
CM10	62.00 ab	59.17
CM7	10.00 c	58.12
<i>S. sclerotiorum</i>	0.00 c	

Means followed by different letters differ significantly according to Tukey's multiple range test ($p \leq 0.05$). Data are means of five replicates with 10 sclerotia per replicate

*data are means of six replicates

Parasitism is the direct utilization of one organism by another. Hoes and Huang (1975) compared PDA medium and sliced carrots system as a method for assaying sclerotia viability during parasitism assessments. They observed that the viability of uncontaminated sclerotia could be assessed with an equal efficiency on either.

However, the viability of moderately contaminated sclerotia were masked on PDA by contaminants that showed faster growth than *S. sclerotiorum*. Viability *per se* is studied on carrot slice because the pathogen growth is not conspicuous on PDA.

In this instance, the assessment of mycoparasitism by *C. minitans* using plant-tissue-based systems such as carrot slices has given excellent results (Cassiolato 1995; Cassiolato *et al.* 1996; Hoes and Huang 1975). Mutants of *C. minitans* showed consistent high infection rates of parasitism of sclerotia, resulting in low viability of sclerotia in fresh sliced carrots, suggesting that these mutants could be successfully used as sclerotial mycoparasites of *S. sclerotiorum*. Three out of five antibiotic producing mutants of *C. minitans* B21 (CM4b, CM4c and CM23) were shown to be destructive parasites of *S. sclerotiorum* by killing more than 80% of sclerotia (Table 1). Parasited sclerotia were unable to colonise carrot slices and were decayed and soft as a result of disintegration by the hyphae. The colonized sclerotia were completely covered by pycnidia (Fig. 1B), with the medullary tissue brown in colour and they collapsed readily with a slight needle pressure. Mutant 4b aggressively colonized sclerotia and penetrated the cell wall (Fig. 1A) as compared to the wild-type.

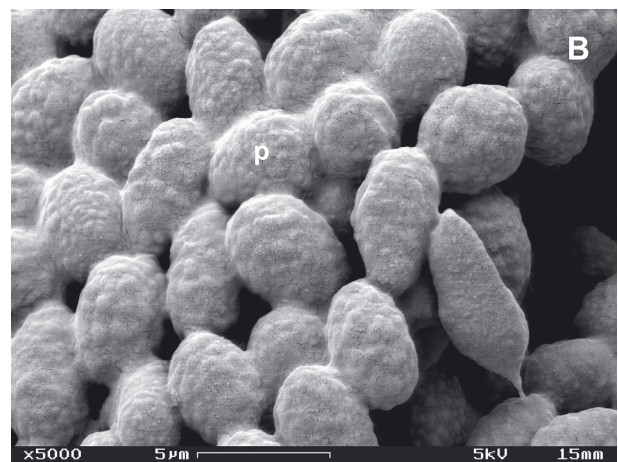
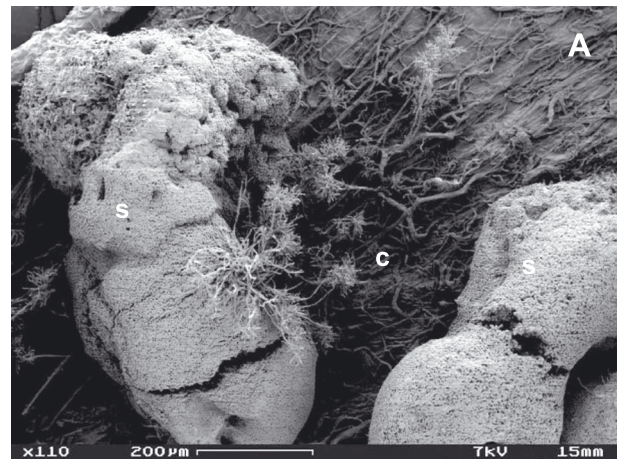


Fig. 1. Parasitism of *S. sclerotiorum* by a mutant of *C. minitans* CM4b viewed by SEM. A) Image shows mycelial colonization of the antagonist on the sclerotia; B) Pycnidia of *C. minitans* on sclerotia. s = *S. sclerotiorum*; c = *C. minitans*; p = pycnidia

Table 2. Size of pycnidiospores of the mutants and effects of temperature on mycelial growth and sporulation of mutants of *C. minitans* B21

Mutants	Size of pycnidiospores* [µm]		Radial growth [cm]			Sporulation (10 ⁶ spores/ml)		
	length	width	18°C	22°C	28°C	18°C	22°C	28°C
CMB21	2.77	2.15	5.28 abAB	5.56 aA	3.97 aB	5.86 abA	7.53 aA	5.58 aA
CM4b	2.94	2.25	5.76 aA	5.88 aA	4.01 aB	5.13 abB	7.95 aAB	11.73 aA
CM4c	2.97	2.40	5.37 aA	4.87 aAB	2.60 bB	9.59 aA	7.00 abA	7.67 aA
CM7	3.74	2.72	5.41 aA	4.97 aA	2.22 bB	9.19 aA	10.79 aA	5.62 abA
CMI0	3.15	2.55	4.13 bAB	4.85 aA	2.91 abB	2.27 bA	9.11 bA	8.81 bA
CM23	2.91	1.95	5.06 abA	5.17 aA	2.68 bB	7.66 abA	9.36 aA	9.58 aA

Means followed by different letters (capital on vertical and small on horizontal) differs significantly according to Tukey's multiple range test ($p \leq 0.05$). Data are means based on three replicates

*data are means of 50 replicates

Penetration of *C. minitans* occurred after the cell wall had started to decompose either naturally or as a result of enzymatic action or other metabolites from the mycoparasite. The biochemical basis for such events is still poorly understood. Before penetrating the host, mycoparasites have been known to use mechanical or enzymatic activities (Sutherland *et al.* 1984; Elad and Misaghi 1985). *Talaromyces flavus* has parasitized *S. sclerotiorum* by direct penetration of the cell wall without formation of appressoria (McClaren *et al.* 1986). When *C. minitans*, *Trichoderma* spp. and *Fusarium solani* were grown in dual culture with *S. sclerotiorum* it was observed morphological abnormalities in hyphae of *S. sclerotiorum* and profuse branching giving withes broom effect (Zazzerini and Tosi 1985).

Temperature is also likely to be an important factor in the design of biocontrol agents screening. On this experiment, the poorest results obtained were on the low and higher temperature conditions. All mutants did not show significant differences from the wild type and exhibited their optimal temperature for growth at 20°C. However, different temperatures did not interfere with sporulation of mutants of *C. minitans* B21, except for CM 4b at 18°C which differed from the others and showed the lowest sporulation (Table 2). These results are similar to that reported in the literature. Trutman *et al.* (1980) and Turner and Tribes (1976) observed that the optimum temperature for germination, growth, infection of sclerotia, and destructive parasitism by *C. minitans* was close to 20°C. Temperatures below 7°C and above 24°C resulted in comparatively slow rates of germination of pycnidiospores and parasitism of sclerotia of *S. sclerotiorum* by *C. minitans*.

The new mutants of *C. minitans* presented variation in regard to morphology, and differences of size (Table 2). All mutants and wild-type exhibited just one nuclei. The mutants produced pycnidiospores greater than the wild-type, and only the mutant CM23 produced pycnidiospores smaller than the wild-type.

The increase in activity of hydrolases such as chitinase is often associated with lysis of hyphal walls of all of phytopathogens. The results of the present investigation showed that the mutagenesis maintained chitinase synthesis (Table 3). The highest production occurred in all mutants evaluated. Elad *et al.* (1982) concluded that

production of lytic enzymes can be resulted as a tool for screening highly parasitic isolates of *Trichoderma* in soil. Based on ultra structural observations and cytochemical localization of N-acetylglucosamine residues, Cherif and Benharmou (1990) provides indirect evidence that chitinase production may have significance in the mycoparasitism of *Trichoderma* on *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

Table 3. Chitinase production in different mutants and wild – type of *C. minitans*

Treatments	Chitinase activity (medium)
Control (chitin solution)	15.9033 a
CMB21	14.4400 b
CM4b	14.3933 b
CM23	14.2933 b

Chitinase activity was expressed by the optical density at 550 nm determined for each mutant extract. Means followed by the same letter are not significantly different from each other, according to Tukey's test ($p \leq 0.05$)

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POLISH SUMMARY

MUTANTY GRZYBA *CONIOTHYRIUM MINITANS* O ZWIĘKSZONEJ SKUTECZNOŚCI W ZWALCZANIU PATOGENA *SCLEROTINIA SCLEROTIORUM*

Grzyb *Coniothyrium minitans* jest nadpasożytem innego gatunku grzyba, *Sclerotinia sclerotiorum* – patogena wielu ważnych gospodarczo roślin uprawnych. W pracy przedstawiono metodę wykorzystania promieni ultrafioletowych (UV) w celu uzyskania mutantów grzyba *C. minitans* o zmodyfikowanym wytwarzaniu enzymu chitynazy, tolerancji na wysokie dawki fungicydu iprodion oraz skutecznych przeciwko patogenowi *S. sclerotiorum*. Spośród 59 zmutowanych izolatów, trzy izolaty inhibowały wzrost grzybni testowanego patogena. Zainfekowanie sklerot grzyba *S. sclerotiorum* przez nowe zmutowane izolaty oceniano na żywym materiale roślinnym (plasterki marchwi). Ponad 80% sklerot zostało zainfekowanych przez zmutowane izolaty oraz izolaty *C. minitans* występujące w naturalnym środowisku. Zmutowane izolaty zachowywały trwałą zdolność do produkcji większych ilości chitynazy oraz wykazywały różnice dotyczące morfologii i zarodnikowania grzyba. Biorąc pod uwagę wyniki prezentowanych badań można wnioskować, że nowe biotypy grzyba *C. minitans* są skuteczne w zwalczaniu patogena *S. sclerotiorum*.