

Biological and Chemical Control of *Sclerotinia sclerotiorum* using *Trichoderma* spp. and *Ulocladium atrum* and Pathogenicity to Bean Plants

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ABSTRACT

Four isolates of *Sclerotinia sclerotiorum* were tested for pathogenicity in IPA-10 variety bean plants (*Phaseolus vulgaris* L.), and all were pathogenic. Biological control in vitro was evaluated using eight isolates of *Trichoderma* spp. and, one of *Ulocladium atrum*. Chemical control in vitro with fungicides Thiophanate methyl, Iprodione and Carbendazim was also tested. Except *U. atrum*, all *Trichoderma* isolates showed antagonistic potential against *S. sclerotiorum*, where isolate 3601 presented the best performance. Thiophanate methyl chemical control was the most efficient. This fungicide and isolate 3601 were compared in vivo in greenhouse. There was statistical difference between the treatments, and the application of fungicide and antagonist before the pathogen was the most efficient approach, reducing the percentage of pathogenicity to 32.94% and 37.04%, respectively.

Key words: *S. sclerotiorum*, *Trichoderma*, antagonist, bean plant.

INTRODUCTION

Several diseases affect the bean plants in Brazil, some causing great damages such as anthracnose, angular leaf spot, fusariosis and white mold caused by *Sclerotinia sclerotiorum* (Lib.) DeBary. The latter has a circle of hosts consisting of 408 species and 278 genera of plants approximately (Boland and Hall, 1994). Cother (2000) observed that the isolates of Australian specimens of *S. sclerotiorum* were pathogenic to 45 plant species comprised in 21 families of native plants from the east Australian coast. Lithourgidis et al (2003) also noticed non-specific host of isolates obtained from

bean and cucumber in leguminous trees, both inducing the symptoms in host under test. This pathogen has been associated to significant loss of commercial yield of crops of beans irrigated at the central region of Brazil (Charchar et al., 1999). In Pernambuco state, the incidence of white mold at the Agreste Meridional region of the state has been observed in small commercial cultivars (Miranda et al., 2002).

This pathogen control through the conventional practice and chemical fungicide usage is the most common method. However, this technique is incredibly expensive and presents a very negative ecological impact due to toxic residues (Rocha and

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Oliveira, 1998). Another prominent practice of management of plant diseases by fungi is the use of biocontrol. Several species of *Trichoderma* have been reported as potential biocontrol agents of phytopathogenic fungi on soil (Chet, 1987), including *S. sclerotiorum* (Trutmann and Keane, 1990; Pereira et al., 1996; Gracia-Garza et al., 1997; Illipronti Júnior and Machado, 1998; Lobo Júnior and Abreu, 2000). Earlier studies have shown *U. atrum* as a hopeful biocontrol agent of some phytopathogenic fungi, such as species of *Botrytis*, mainly *B. cinerea* (Kohl et al., 2000; Boff et al., 2001). However, studies about the efficiency of *U. atrum* as potential biocontrol agent of *S. sclerotiorum* are very scarce, and only the works by Li et al. (2003) are available about this topic.

Considering the difficulties of controlling *S. sclerotiorum*, the present work aimed to study the possibility of biological control of *S. sclerotiorum* in bean plants by species of *Trichoderma* and *Ulocladium atrum*, providing resources for further studies on biocontrol in field conditions.

MATERIALS AND METHODS

Isolates of *S. sclerotiorum*, *Trichoderma* spp. and *U. atrum* used in the study

Four isolates of *S. sclerotiorum* and nine isolates of antagonists from different localities, hosts and substrates were used in this study as shown in Table 1.

Table 1 - Isolates of *Sclerotinia sclerotiorum* and antagonists *Trichoderma* spp. and *Ulocladium atrum*

Species	Isolate	Origin	Substrate
<i>S. sclerotiorum</i>	806 (micoteca - URM)	USA	soil
<i>S. sclerotiorum</i>	Ss5 (EMBRAPA- rice and beans)	Rio Grande do Sul- Brazil	soy
<i>S. sclerotiorum</i>	Ss17 (EMBRAPA- rice and beans)	Distrito Federal- Brazil	lettuce
<i>S. sclerotiorum</i>	Ss 11 (micoteca - URM)	Pernambuco- Brazil	beans
<i>T. viride</i>	2745 (micoteca - URM)	Pernambuco- Brazil	sugarcane
<i>T. viride</i>	2820 (micoteca - URM)	Alagoas- Brazil	sugarcane
<i>T. harzianum</i>	3601 (micoteca - URM)	Paraná- Brazil	Soil
<i>U. atrum</i>	3180 (micoteca - URM)	Pernambuco- Brazil	barley
<i>T. aureoviride</i>	4912 (micoteca - URM)	Pernambuco- Brazil	bean rhizosphere
<i>T. aureoviride</i>	4913 (micoteca - URM)	Pernambuco- Brazil	bean rhizosphere
<i>T. aureoviride</i>	4924 (micoteca - URM)	Pernambuco- Brazil	bean rhizosphere
<i>T. aureoviride</i>	4915 (micoteca - URM)	Pernambuco- Brazil	bean rhizosphere
<i>T. aureoviride</i>	4916 (micoteca - URM)	Pernambuco- Brazil	bean rhizosphere

Pathogenicity Test of isolates of *S. sclerotiorum* on bean plants

Two types of pathogen inoculum were prepared using cultures of *S. sclerotiorum* grown for 10 days on PDA (Potato Dextrose Agar) medium at 28° C. The first type of inoculum consisted of sclerotia and the second one was a suspension of mycelium-agar triturated with sterilized distilled water.

P. vulgaris plants of variety IPA-10 were cultivated in plastic pots containing 3 kg of soil (sandy clay loam soil) sterilized with methyl bromide. The inoculation was carried out after 15 days of planting. The sclerotia were set at a depth of 3 cm in the soil and 2 cm far from the stalk, each plant receiving three sclerotia. The inoculum suspension was also applied to the soil next to the roots at a depth of 5 cm, and each plant received 20 mL of inoculum. The controls received 20 mL of sterilized distilled water without inoculum of *S.*

sclerotiorum. All plants were kept in greenhouse conditions and the experimental design was completely randomized with ten repetitions for each treatment.

The plant symptoms of disease isolates of pathogen were evaluated at 10, 15, 20 and 25 days after inoculation and consisted of observing typical symptoms according to Hall and Phillips (1996). The data were submitted to statistical tests to compare isolates. A model of binomial multivariate analyses was applied according to GEE methodology (Prentice and Zhao, 1991) with structure of permutable correlation to better explain the percentage of leaves, petioles and stalks infected.

S. sclerotiorum Biological Control *in vitro* with isolates of *Trichoderma* spp. and *U. atrum*.

The rate of mycelium growth of fungal isolates was calculated by daily measurements of the

diameter of the colonies in two opposite directions using a millimeter ruler for six days.

The matching method between the antagonist and phytopathogen described by Dennis and Webster (1971) was used to determine the antagonistic potential of isolates of *Trichoderma* and *U. atrum* against the isolates of *S. sclerotiorum*. The method consisted of inoculating the Petri dishes containing PDA, with disks of mycelium-agar from the phytopathogen and antagonists on halfway points, 7 cm apart from each other, and 1 cm away from the edge of the plate. Then was considered the rate

of mycelium growth of each isolate in such a way that the colonies could reach simultaneously the center of the plate. Daily measures were carried out in opposite directions until the meeting of the two mycelia and/or until one of the two fungi were overlaid by the other.

Ranking based on Bel et al. (1982) scale was used in order to evaluate the antagonistic potential of isolates of *Trichoderma* and *U. atrum* and interactions between the isolates were observed under optical microscopy (Table 2).

Table 2 - Classes of antagonism to *Trichoderma* and *U. atrum* with *S. sclerotiorum*, adapted from Bell et al. (1982).

Class	Characteristic
1	<i>Trichoderma</i> or <i>Ulocladium</i> grows and covers completely all colonies of <i>S. sclerotiorum</i> and medium surface.
2	<i>Trichoderma</i> or <i>Ulocladium</i> grows and covers 2/3 of medium surface.
3	Antagonists and phytopathogen colonize each one, half of the medium surface and no one seems to dominate the other.
4	<i>S. sclerotiorum</i> colonizes 2/3 of medium surface.
5	<i>S. sclerotiorum</i> grows and covers completely all colonies of <i>Trichoderma</i> or <i>Ulocladium atrum</i> and the medium surface.

Chemical Control of *S. sclerotiorum* in vitro

Three fungicides were selected for the present study: Thiophanate methyl, Carbendazim, and Iprodione. These were incorporated into PDA medium, following Caldari Junior (1998) in four different concentrations of the active ingredient (1, 10, 50 and 100ppm). Afterwards, discs of mycelium-agar with isolates of the phytopathogen were inoculated in medium containing fungicides, with four repetitions for each treatment; including control inoculated in fungicide-free medium and maintained at 28° C.

Mycelial growth was measured at regular intervals (24 h) to evaluate the chemical control and alterations in macroscopic aspects of colonies. The data were submitted to statistical analysis in order to compare the efficiency of fungicides.

The experiment was arranged in a complete randomized 4x3x4 factorial scheme represented by four isolates of pathogen; three fungicides; four concentrations of active ingredient with four repetitions for each treatment, and control. Variance analysis and means were compared using Tukey test at 5% of probability.

Comparison between biological and chemical control in bean plants in vivo.

The best performance *in vitro* of *Trichoderma* isolate for biocontrol and the most efficient fungicide in chemical control of *S. sclerotiorum* were both selected and tested *in vivo* in greenhouse. Bean seeds were seeded in pots containing sterilized soil with methyl bromide. After 15 days of plant growth, phytopathogen and then antagonist and fungicide were employed in different treatments, except in one, where the antagonist was inoculated before the pathogen. The different treatments are shown in Table 3. The antagonist was added to the soil of the pots with bean plants, being introduced in autoclaved rice as described by Noronha et al. (1996), in a concentration of 2g of inoculum/kg. The fungicide used in this phase of the study was applied as recommended by manufacturer (powder diluted in water sprinkled on the plants). The evaluation of the results was carried out as described above when testing the pathogenicity.

Table 3 - Treatment scheme and application time of antagonist and fungicide selected to compare chemical and biological control of *Sclerotinia sclerotiorum*.

Treatment	Inoculation time/ antagonist or fungicide application
3601xSs11	antagonist employed 8 days before the pathogen.
Ss11=3601	antagonist employed at the same time as the pathogen
Ss11x3601	antagonist employed 8 days after the pathogen.
Ss11x thiophanate methyl	fungicide employed as recommended by manufacturer

RESULTS AND DISCUSSION

Pathogenicity of *S. sclerotiorum* to bean plants

The first symptoms of white mold were observed on the first evaluation, on tenth day after inoculations took place. The leaves turned yellow, wilted and presented humid lesions on petioles, branches and stalks in advanced stage culminating in plant death, with the presence of white mycelium and esclerotia in stalks, branches and immature beans. Symptoms were considered as being severe for four tested isolates, 25 days after inoculation, contrasting with the plants used as control, which showed no symptoms. The four isolates tested for pathogenicity were isolated again using plants with symptoms and were identical to those seen in macroscopic and microscopic observation.

All the tested isolates were pathogenic to bean

plants. Isolates Ss11, Ss17 and 806 induced the most severe disease symptoms, with 89.4, 87.2 and 89.8% of symptoms, respectively, whereas isolate Ss5 showed the lowest percentage of symptoms with 76.5% (Table 4). Statistical analyses of the data showed differences between the isolates, and only isolate Ss5 was significantly less pathogenic than the other ones (Table 5). The isolates caused pathogenicity, which was possible due to the suitable climate conditions during the experiment, where the average maximum and minimum temperatures were 33.2 and 22°C respectively, and the maximum and minimum relative humidity values 90.8 and 47.7% respectively. Bianchini et al. (1997) stated that mild temperatures associated with high humidity were essential factors to the development of plant damages caused by *S. sclerotiorum*.

Table 4 - Percentage of pathogenicity presented by isolates of *Sclerotinia sclerotiorum* in *Phaseolus vulgaris* plants.

Isolate	Pathogenicity to <i>Phaseolus vulgaris</i> (%)
Ss11	89.4
806	89.8
Ss17	87.2
Ss5	76.5

Table 5 - Comparison between treatments and their respective statistics for percentages of pathogenicity of *Sclerotinia sclerotiorum* in *Phaseolus vulgaris* plants.

Treatments	Statistical Correspondents
Control x Ss11	2.885827 x 0.00000000*
Control x Ss17	2.885828 x 0.00000000*
Control x Ss5	2.885827 x 0.00000000*
Control x 806	2.885829 x 0.00000000*
Ss11 x Ss17	2.725556 x 0.60162255
Ss11 x Ss5	3.095548 x 0.07850673*
Ss11 x 806	1.024242 x 0.91938787
Ss17 x Ss5	3.605397 x 0.05759230*
Ss17 x SsM	4.622716 x 0.49656424
Ss5 x 806	4.992292 x 0.02546046*

*Significant at 0.10 level of probability. Statistical correspondents of Waldo f matrix of covariance were calculated to determine statistical differences between treatments.

Bolland (1997) reported the existence of a large range of hosts to *S. sclerotiorum*. Charchar et al (1999) tested isolates obtained from cotton in different species of hosts such as: bean, okra and cotton plants and observed that all were infected by the phytopathogen. Cother (2000) observed that the isolates of native Australian fungi were pathogenic to 45 plant species in 21 families of native plants from the Australian east coast. Lithourgidis et al (2003) also noticed the host non-specificity of isolates obtained from bean and cucumber in leguminous trees, both inducing symptoms in host under test.

This study also observed that the pathogen was not specific, therefore the isolates tested in beans from different origins of host presented very close percentages of pathogenicity, and isolate 806 showed the largest percentage of pathogenicity (89.8%) and Ss5 the lowest one (76.5%).

Antagonistic potential of isolates of *Trichoderma* spp. and *Ulocladium atrum* against *Sclerotinia sclerotiorum* in vitro

Table 6 - Classes of antagonism adapted from Bell et al. (1982) for isolates of *Trichoderma* spp and *Ulocladium atrum* against isolates of *Sclerotinia sclerotiorum*.

Class of antagonism	Isolate
1	3601, 4915, 4916
2	2820, 2745, 4912, 4913, 4914
3	3180

Besides mycelium growth, the level of sporulation of antagonist isolate was also observed. The production of spores of isolates 3601, 4914, 4915, 4916 and 3180 was not affected when in contact with pathogen, but isolates 4912 and 4913, presented a slight decrease in sporulation, while isolates 2745 and 2820 hardly produced spores. Batista (2002) reported that the process of sporulation would be possibly a favorable characteristic to antagonists, therefore, new inoculum was desirable in the presence of pathogen, inhibiting its actions, due to greater density of antagonist inoculum.

The inhibition of mycelium growth of *S. sclerotiorum* was more intense with isolates 2745 (*T. viride*), 2820 (*T. viride*), 4912 (*T. aureoviride*), 4913 (*T. aureoviride*) and 4914 (*T. aureoviride*) that colonized 2/3 of the plate without growing over the pathogen. This inhibition was probably related to the capacity of production of antibiotic substances by these antagonists, which could

Of the nine antagonist isolates evaluated for potential biocontrol of *S. sclerotiorum*, only three showed satisfactory action (3601, 4915 and 4916), being from class 1 of antagonism, adapted from Bell et al. (1982). The other isolates of *Trichoderma* spp. presented mild antagonistic potential, pertaining to class 2 of antagonism. Isolate 3180 (*U. atrum*) didn't show antagonistic action to *S. sclerotinia sclerotiorum* (Table 6). Li et al. (2003) reported the existence of antagonistic potential of *U. atrum* against *S. sclerotiorum*, although this antagonistic effect was not observed in this work. In the present study the only tested isolate of *U. atrum* didn't show antagonistic potential against the pathogen. This could be due to the preservation of the isolate, which was obtained from the Micoteca URM collections. According to Smith and Onions (1994) the preservation might microorganisms may alter or inactivate important physiological characteristics such as pathogenicity, sporulation or enzymes liberation and other compounds.

affect the pathogen development (Chet and Baker, 1981; Blakeman and Fokkema, 1982; Papavizas, 1985). Campbell (1989) reported some effects caused by antibiotic substances liberated by antagonists such as reduction or paralysis of mycelium growth and sporulation, reduction in spore germination, besides distortions on hyphae and plasmolysis.

Microscopic observation of the interaction area between the colonies of antagonists and phytopathogen showed morphological alterations such as: parallel growth of antagonist and of pathogen, formation of hyphae rings, with rolling up of hyphae, hyphae fragmentation, mycelium without protoplasmatic content and the penetration of the hyphae from *S. sclerotiorum* by all *Trichoderma* isolates.

Elad (2000) related the consecutive stages involving mycoparasitism of pathogenic fungi by species of *Trichoderma*, such as: chemotrophic growth (where exudates of pathogen attract

antagonist); recognition between the phytopathogen and antagonist; adhesion to the pathogen hyphae and finally, degradation, caused by the secretion of enzymes such as proteases and chitinases to degrade and penetrate in the pathogen cell wall. According to Brunner et al. (2003), the enzyme 73-KDA N-acetyl- β -D-glucosaminidase was related to genes involved in induction of chitinase, and this enzyme was the most relevant for biocontrol because was related with the degradation of fungi cell wall.

The antagonist isolates presented rings of hypha when matched with the pathogen. Elad et al. (1987), studying biological control of *Rhizoctonia solani* by *Trichoderma* spp., observed that these rings were frequent during matching and then related this structure to parasitism. However, Rocha and Oliveira (1998) observed that even lacking the mycelium from the host, in this case *Colletotrichum gloeosporioides*, there were rings of hyphae formed by *Trichoderma*, suggesting that

these structures were not directly related to mycoparasitism.

Chemical control *in vitro* of isolates de *S. sclerotiorum*.

The three tested fungicides were able to reduce significantly the mycelium growth of four isolates of *S. sclerotiorum*. Thiophanate methyl showed the best results even at lower concentration of active ingredient (1ppm), affecting considerably the mycelium growth of pathogen and at other concentrations tested (10, 50 and 100 ppm) no growth of pathogen was observed. For other fungicides the concentration of the active ingredient able to inhibit the pathogen growth varied, with 10 ppm for Iprodione and 50 ppm for Carbendazim (Table 7). All of the *S. sclerotiorum* isolates analyzed here were not able to grow at a concentration of 100 ppm of all the tested fungicides.

Table 7 - Effect of fungicides in different concentrations of active ingredient on mycelium growth of *Sclerotinia sclerotiorum*, after 144 hours in BDA medium.

Isolate	Fungicides		
	Iprodione	Thiophanate methyl	Carbendazim
Ss5	33.6058 bB ¹	18.3200 cC	44.7227 bA
SsM	34.6774 aB	22.5358 bC	46.6329 aA
Ss11	33.4899 bB	18.4951 cC	45.3339 bA
Ss17	33.3406 bB	25.0294 aC	45.1590 bA

¹Mean of four replications (cm). Means transformed by $y=\sqrt{x+1}$, followed by the same letter did not differ at 5% of probability in column (capital letters) or in line (lower-case letters) by Tukey test

Mueller et al. (2002) studied the efficiency of Thiophanate methyl and other fungicides in chemical control of *S. sclerotiorum* and showed that this fungicide was efficient in chemical control of the pathogen at 7 μ g/mL. Kimura et al (2001) tested 19 fungicides including Iprodione and Thiophanate methyl against *Botrytis cinerea* and proved that iprodione and procimidone were the most efficient against this pathogen overcoming results obtained for Thiophanate methyl. Matheron and Matejka (1989) compared several fungicides including iprodione and stated that although its efficacy against *S. sclerotiorum* tends to a mild effect, becoming stable action at high concentrations. Studies of Breneman et al (1987) and Poter and Philipps (1985) showed the resistance of *S. minor* to several fungicides of the group of dicarboxamidas, including iprodione and

vinclozolina. Vital (1990) observed the growth of *Sclerotium coffeicola* in concentrations until 2000 ppm of iprodione, which would make this control method inappropriate.

Comparison of chemical and biological control *in vivo*.

All the treatments were statistically significant in relation to controls. The comparisons between statistical correspondents indicated that treatments 3601XSs11 and Ss11XThiophanate methyl were the most efficient in the chemical control of *S. sclerotiorum*, decreasing the symptoms induced by pathogen in more than 30% in relation to controls that presented high levels of incidence (87.25%). The treatments Ss11=3601 and Ss11X3601 reduced the levels of incidence of white mold 16.21 and 7.05%, respectively (Fig. 1).

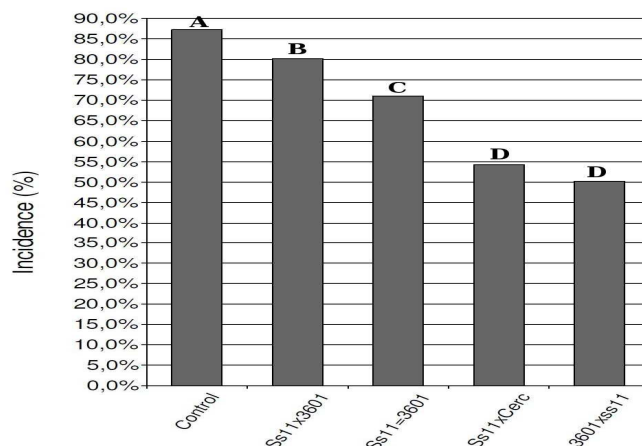


Figure 1 - Incidence levels of white mold caused by *Sclerotinia sclerotiorum*, in different treatments. Means followed by the same letters did not differ at the 10% level of the probability.

These results suggested a correlation between time of application of antagonist and the best control of pathogen. As soon as the antagonist is applied, the better is the performance against the pathogen. Jackish (1996) used similar methods and observed that no treatment had desirable action of *Trichoderma* spp., except when *T. koningii* was applied 24 h before the pathogen. Rocha and Oliveira (1998) found similar results studying the action of *Trichoderma* spp. against *Colletotrichum gloeosporioides* in passion fruit tree. Bolland (1997) tested several agents of biocontrol, including *T. viride* and fungicide benomyl, for controlling white mold in bean plants and noticed no significant difference between these two forms of control. Both the treatments were efficient; however, such results were observed when environmental conditions were not favorable to the development of disease. Cardoso et al. (1997) observed in comparative studies between chemical and biological controls of root-rot of bean plants that *Trichoderma* sp. was not efficient when compared to some fungicides tested. Pereira et al. (1996) held forth integral control of *S. sclerotiorum*, showing solarization as one of the most efficient way of controlling this pathogen, followed by application of species of *Trichoderma*. However, chemical control with

Iprodione was inefficient, even in other associations with other forms of control.

The reduction in the incidence of diseases by using chemical control with Thiophanate methyl was considered low. Regarding the results obtained *in vitro* and greenhouse, this low reduction would be more consistent in field experiments, on natural soil, where other chemical, and mainly biological factors, could interfere in this kind of control (Kimati, 1995). The form of fungicide application (conventional application) could be another factor to be considered when analyzing this low level of pathogen control. Oliveira *et al.* (1995) and Vieira *et al.* (2003) stated the importance of application know-how in relation to fungicides used to control the white mold, reinforcing the evident efficiency of fumigation and chemigation in detriment of compared to conventional application methods.

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RESUMO

Quatro isolados de *Sclerotinia sclerotiorum*, foram testados quanto à patogenicidade em plantas de feijão, variedade IPA-10 sendo que todos se mostraram patogênicos. Foram avaliados o controle biológico e químico *in vitro*, utilizando-se oito isolados de *Trichoderma* e um de *Ulocladium atrum*, e o controle químico *in vitro*, com os fungicidas Tiofanato metílico, Iprodione e Carbendazim. Com exceção de *U. atrum* todos os isolados dos antagonistas mostraram potencial antagonístico contra *S. sclerotiorum*, destacando-se o isolado 3601 como o de melhor desempenho. No controle químico, Tiofanato metílico foi o mais eficiente, sendo este fungicida e o isolado 3601 comparados *in vivo* em casa-de-vegetação. Foram observadas diferenças estatísticas entre os tratamentos, sendo que a aplicação do fungicida e do antagonista antes da introdução do patógeno foi mais eficiente, com redução do percentual de incidência em 32,94% e 37,04%, respectivamente.

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