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 cost. 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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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.

| Species        | Isolate | Origin                  | Substrate       |
|----------------|---------|-------------------------|-----------------|
| *S. sclerotiorum* | 806 (micoteca - URM) | USA                     | soil            |
| *S. sclerotiorum* | Ss5 (EMBRAPA- rice and beans) | Rio Grande do Sul- Brazil | soy             |
| *S. sclerotiorum* | Ss17 (EMBRAPA- rice and beans) | Distrito Federal- Brazil | lettuce         |
| *S. sclerotiorum* | Ss 11 (micoteca - URM) | Pernambuco- Brazil       | beans           |
| *T. viride*     | 2745 (micoteca - URM) | Pernambuco- Brazil       | sugarcane       |
| *T. viride*     | 2820 (micoteca - URM) | Alagoas- Brazil          | sugarcane       |
| *T. harzianum*  | 3601 (micoteca - URM) | Paraná- Brazil           | Soil            |
| *U. atrum*      | 3180 (micoteca - URM) | Pernambuco- Brazil       | barley          |
| *T. aureoviride* | 4912 (micoteca - URM) | Pernambuco- Brazil       | bean rhizosphere |
| *T. aureoviride* | 4913 (micoteca - URM) | Pernambuco- Brazil       | bean rhizosphere |
| *T. aureoviride* | 4924 (micoteca - URM) | Pernambuco- Brazil       | bean rhizosphere |
| *T. aureoviride* | 4915 (micoteca - URM) | Pernambuco- Brazil       | bean rhizosphere |
| *T. aureoviride* | 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 claim 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
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**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     | *Trichoderma* or *Ulocladium* grows and covers completely all colonies of *S. sclerotiorum* and medium surface. |
| 2     | *Trichoderma* or *Ulocladium* 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     | *S. sclerotiorum* colonizes 2/3 of medium surface. |
| 5     | *S. sclerotiorum* grows and covers completely all colonies of *Trichoderma* or *Ulocladium atrum* 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 100 ppm). 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 2 g 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 Phaseolus vulgaris (%) |
|---------|----------------------------------------|
| 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 (89.8%) and Ss5 the lowest one (76.5%).

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

| 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 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
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 (1 ppm), 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 | Iprodione | Thiophanate methyl | Carbendazim |
|---------|------------|--------------------|-------------|
| Ss5     | 33.6058 bB | 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  |

*Mean 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 µg/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 Brenneman 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).
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 gloeosporiodes* 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 da aplicação 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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