Selection of *Trichoderma* spp. strains for the control of *Sclerotinia sclerotiorum* in soybean

Patrícia Elias Haddad[1], Luis Garrigós Leite[2], Cleusa Maria Mantovanello Lucon[1] and Ricardo Harakava[1]

[1]Instituto Biológico, Avenida Conselheiro Rodrigues Alves, nº 1.252, Vila Mariana, CEP 04114-900 São Paulo, SP, Brazil. E-mail: patriciaehaddad@yahoo.com.br, mantova@biologico.sp.gov.br, harakava@biologico.sp.gov.br [2]Instituto Biológico, Centro Experimental, Alameda dos Vidoeiros, nº 1.097, Gramado, CEP 13101-680 Campinas, SP, Brazil. E-mail: lgleite@biologico.sp.gov.br

Abstract – The objective of this work was to evaluate, in vitro and in vivo, the potential of *Trichoderma* spp. strains to control *Sclerotinia sclerotiorum* in soybeans (*Glycine max*) and to perform the molecular identification of the best performing strains. The effect of 120 strains of *Trichoderma* spp. on the viability of *S. sclerotiorum* sclerotia was evaluated in vitro through immersion in suspension of conidia from the antagonists and plating in culture medium. The best performing strains were evaluated in vivo, in a greenhouse, for control of the pathogen inoculated on 'Pintado' soybean seeds and plants. Of the 120 strains tested in vitro, 22 strains of *Trichoderma* spp. caused 100% inhibition of sclerotia germination. In the greenhouse, five strains inhibited the negative effect of the pathogen on seed germination and two strains increased in up to 67% plant dry matter. The best performing strains were identified as *T. koningiopsis* (3 strains), *T. asperelloides* (3), *T. atroviride* (2), and *T. virens* (1). *Trichoderma* strains are able to protect soybean plants from the harmful effect of *S. sclerotiorum* and, at the same time, they can promote the growth of the aerial part in greenhouse conditions.

Index terms: *Glycine max*, biological control, DNA sequencing, sclerotia, white mold.

Seleção de estirpes de *Trichoderma* spp. para o controle de *Sclerotinia sclerotiorum* em soja

Resumo – O objetivo deste trabalho foi avaliar, in vitro e in vivo, o potencial de isolados de *Trichoderma* spp. no controle de *Sclerotinia sclerotiorum* em soja (*Glycine max*) e identificar molecularmente as estirpes que mais se destacaram. O efeito de 120 isolados de *Trichoderma* spp. sobre a viabilidade de escleródios de *S. sclerotiorum* foi avaliado in vitro, por imersão em suspensão de conídios dos antagonistas e plaqueamento em meio de cultura. As estirpes que mais se destacaram foram avaliadas, in vivo, em casa de vegetação, no controle do patógeno em plantas e sementes de soja 'Pintado'. Dos 120 isolados testados in vitro, 22 inibiram em 100% a germinação de escleródios. Em casa de vegetação, cinco estirpes inibiram os efeitos prejudiciais do patógeno na germinação das sementes e duas estirpes proporcionaram aumento de até 67% na massa de matéria seca das plantas. As melhores estirpes foram identificadas como *T. koningiopsis* (3 estirpes), *T. asperelloides* (3), *T. atroviride* (2) e *T. virens* (1). *Trichoderma* spp. conseguem proteger plantas de soja 'Pintado' do efeito prejudicial de *S. sclerotiorum* ao mesmo tempo em que podem promover o crescimento da parte aérea em condições de casa de vegetação.

Termos para indexação: *Glycine max,* controle biológico, sequenciamento de DNA, escleródios, mofo branco.

Introduction

Soybean [*Glycine max* (L.) Merr.] is the most important oleaginous plant cultivated. In Brazil, this crop is one of the main agricultural commodities that contributes to a positive commercial balance. Diseases, such as white mold caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, are one of the main limiting factors that limit full productivity. *S. sclerotiorum* is a pathogen of more than 400 plant species, including several of economic importance (Abdullah et al., 2008). This fungus strives in mild temperatures and high humidity conditions and survives in soil and crop remains through its resistant-structure sclerotia. Control methods involve cultural practices, use of resistant cultivars, and chemical control (Bardin & Huang, 2001). Alternative methods such as biological control, are also being employed and must be used when sclerotia are in a resting state in the soil or in the germinating

stage, when the pathogen is most vulnerable in the environment (Zancan et al., 2012).

Trichoderma spp. consists of a large group of fungi important for biological control. They are found in most ecosystems as plant root colonizers and display several mechanisms that can be used to control phytopathogens such as antibiosis, mycoparasitism, competition, and induction of plant resistance (Consolo et al., 2012). Several species of *Trichoderma* have shown promising results for control of *S. sclerotiorum* (Smith et al., 2013; Tančić et al., 2013; Smolińska et al., 2016). *Sclerotinia sclerotiorum* and *Sclerotinia cepivorum* had their growth inhibited in vitro in up to 81.5%, and cucumber plants could be protected against *S. sclerotiorum* in greenhouse trials using *Trichoderma* strains (Ethur et al., 2005; Hernandez Castillo et al., 2011). These results have drawn scientific attention to *Trichoderma* spp. during the last decades and conferred it broad acceptance as a biological control agent (Schuster & Schmoll, 2010). The demand for biological control agents has increased significantly in recent years (Lucon, 2008), sharing the need for further investigation to provide more options for the control of plant diseases, including the highly impacting white mold disease of soybean.

The objective of this work was to evaluate, in vitro and in vivo, the potential of *Trichoderma* spp. strains to control *Sclerotinia sclerotiorum* in soybeans and to perform the molecular identification of the best performing strains.

Material and Methods

The experiments were carried out at the phytopathological biochemistry laboratory of Instituto Biológico, located in the munipality of São Paulo, in the state of São Paulo, Brazil, between January 2013 and February 2014. One hundred and twenty strains of *Trichoderma* spp., from the Mario Barreto Figueiredo fungi collection of Instituto Biológico, were subjected to the in vitro assay. Inocula of *Trichoderma* spp. strains were produced on autoclaved rice in plastic bags incubated for seven days at 25±2ºC under a 12-hour photoperiod. The LQC 122 *S. sclerotiorum* strain was obtained from the culture collection of Embrapa Meio Ambiente. Sclerotia of the pathogen were multiplied in maize flour-enriched carrot medium (Ferraz & Café Filho, 1998) for 30 days at 21ºC.

For the in vitro inhibiton of sclerotia germination assay, sclerotia of the pathogen were submersed in conidial suspensions of strains of *Trichoderma* spp. ($1x10^7$ conidia per mL) for 30 min. Treated sclerotia were placed on moistened autoclaved filter paper inside Petri dishes and incubated at 25±2ºC during one week under a 12-hour photoperiod. After incubation, sclerotia were surface sterilized by immersion in 50% ethanol for 3 min and 1% sodium hypochlorite for 3 min, followed by three washes in autoclaved distilled water, and placed on BDA medium for incubation during another week at 25±2ºC. The control treatment consisted of submersion of sclerotia in autoclaved distilled water only. The experimental design was completely randomized with three replicates of five sclerotia each. The effect on the pathogen was evaluated by counting the number of viable sclerotia, which were able to germinate within eight days after plating on BDA (Görgen et al., 2009). The 22 strains of *Trichoderma* spp. that caused in 100% inhibiton of the pathogen in the first trial were tested again three more times.

The effect of the strains of *Trichoderma* spp. on soybean seeds germination was evaluated by in vitro and in vivo assays. In the in vitro assay, nine strains of *Trichoderma* spp. were randomly selected among the 22 strains selected previously. 'Pintado' soybean seeds were submersed in conidial suspensions at the concentrations of 10^6 and 10^7 per mL for a few minutes and incubated on filter paper moistened with autoclaved distilled water inside Petri dishes. The control treatment consisted of seeds submersed in autoclaved distilled water only. Each strain was considered as one treatment, with three replicates of 20 seeds in each. Plates were incubated at 25±2ºC during six days under a 12-hour photoperiod. After incubation, germinated seeds were counted for each treatment/strain.

For the in vivo assay in the greenhouse, four strains of *Trichoderma* spp. were randomly selected. These were grown on autoclaved rice grains and incorporated into 700 g of autoclaved commercial substrate Tropstrato Hortaliça 2 (Vida Verde, São Paulo, SP, Brazil) in plastic pots. Three concentrations of *Trichoderma* spp. inocula were evaluated: 0.25, 0.5, and 1.0% (w/v). Each treatment/strain had five replicates (pots) with three seeds each. The control treatment consisted of autoclaved rice grains without *Trichoderma* spp. Pots were kept in the greenhouse under natural conditions,

and germinated seeds were counted 4, 5, and 6 days after sowing. Both in vitro and in vivo assays had a completely randomized design.

The biological control assays were conducted in the greenhouse between June 2013 and February 2014. Nine strains of *Trichoderma* spp. were employed: IB 10/12, IB 07/01, IB 30/12, IB 37/12, IB 62/12, IB 91/12, IB 103/12, IB 111/12, and IB 119/12. The pathogenicity of the LQC 122 *S. sclerotiorum* strain was verified by inoculation of the third trifoliate removed from 'Pintado' soybean plants. Leaves were kept on moistened filter paper in Petri dishes, and a 5-mm mycelium plug of *S. sclerotiorum* was placed on the adaxial surface of the middle leaf next to the midvein. Plates were incubated at room temperature in the dark, and the presence of lesions was checked 48, 66, and 90 hours after inoculation. Ten replicates per plate were evaluated. The control treatment consisted of inoculation with the plug of BDA medium without fungal growth.

For the biological control assay, plastic pots containing 800 g of commercial substrate (Tropstrato Hortaliça 2) were infested with 16 g of *S. sclerotiorum* inoculum and kept in moist chamber for seven days at 22ºC. After this period, 16 g of rice grains colonized by each strain of *Trichoderma* spp. were incorporated to the infested substrate and six 'Pintado' soybean seeds were planted in each pot, with five replicates per pot for each strain. The control treatments consisted of plants inoculated with the pathogen only (infected control) and plants treated with rice grains without *Trichoderma* spp. and without pathogen (untreated control). One week after sowing, three plantlets were removed from each pot. The effect of strains of *Trichoderma* was evaluated by counting germinated seeds one week after sowing, and by determining the incidence of white mold and the weight of above-ground dry matter 35 days after sowing. Root dry matter was not measured since above-ground development reflects root system condition (Björkman et al., 1998). Experiments were repeated three times, data were subjected to analysis of variance and compared by the Scott-Knott test, at 5% probability, using the Assistat software, version 7.5 Beta (Silva & Azevedo, 2006).

At the end of the assay, the ability of the strains of *Trichoderma* spp. to colonize plant roots was verified. Three roots were randomly collected from each treatment, and washed with tap water; 2-cm fragments were incubated on BDA medium amended with antibiotics [0.17 g ampicillin, 0.05 g of pentabiotico (commercial mix of benzylpenicillin and streptomycin) for 1 L of medium] and 0.01% Triton X100 to limit colony expansion. Positive colonization was observed by visualization of *Trichoderma* spp. growth along the root fragments.

Nine strains of *Trichoderma* spp. were subjected to molecular identification by sequencing part of the translation elongation factor (TEF) gene. Polymerase chain reaction (PCR) was performed with primers (5' – CAAAATGGGTAAGGAGGASAAGAC – 3') and tef997R (5' – CAGTACCGGCRGCRATRATSAG – 3') (Shoukouhi & Bisset, 2009). The PCR amplification program consisted of initial denaturation at 94°C per 2 min followed by 40 cycles of 94°C per 30 s – 54°C per 30 s – 72°C per 60 s, and final extension at 72°C per 4 min. Sequencing reaction was performed with the Big Dye 3.1 reagent (Applied Biosystems Inc., Foster City, CA, USA) and analyzed in a capillary automatic sequencer ABI 3500XL (Applied Biosystems, Foster City, USA). Sequences deposited in the GenBank by authors of recognized reputation in *Trichoderma* taxonomy were retrieved for construction of a phylogenetic tree. This tree was constructed by the maximum likelihood method based on Kimura 2-parameter model with 1000 bootstrap replicates using the Mega software, version 6.0 (Tamura et al., 2013).

Results and Discussion

The LQC 122 *Sclerotinia sclerotiorum* strain was pathogenic to 'Pintado' soybean. Symptoms were first observed on trifoliate leaves 48 hours after inoculation and increased progressively with time. Pathogenicity results are in alignment with the observations of Garcia & Juliatti (2012).

Sixty six out of the 120 strains of *Trichoderma* spp. tested have shown the ability to reduce sclerotia germination more than 50% and 22 inhibited germination completely. Similar results were obtained by Smith et al. (2013), who, among 22 strains of *Trichoderma* spp. observed sclerotia inhibition rate of 75 to 80%. Görgen et al. (2009) considered the colonization of *S. sclerotiorum* sclerotia by *T. harzianum* as a form of parasitism and classified sclerotia that did not show myceliogenic germination as unviable. Abdullah et al. (2008) also attributed

the inhibition germination of *S. sclerotiorum* to the mycoparasitism of *T. harzianum*. Mycoparasitism is a complex mechanism triggered by the presence of phytopathogenic fungus, envolving the secretion of hydrolytic enzymes, mainly chitinases, glucanases, and proteases that allow *Trichoderma* spp. hyphae to penetrate hyphae of the host (Reithner et al., 2011).

No significant differences were observed when the two concentrations of *Trichoderma* spp. inocula, 10^6 and 10^7 conidia per mL, were compared in vitro seed germination inhibition test, except for strains IB 69/12 (lower germination at 107) and IB 91/12 (higher germination at 107) (Figure 1). Four strains, IB 10/12, IB 37/12, IB 62/12, and UB 91/12, decreased seed germination in at least one of the inoculum concentrations. In the in vivo seed germination inhibition test performed in pots with substrate, none of the strains interfered in seed germination six days after sowing, although a delaying effect could be observed for some strains the fourth and fifth days (Figure 2). Ousley et al. (1993) observed that some strains of *Trichoderma* spp. can inhibit lettuce (*Lactuca sativa*

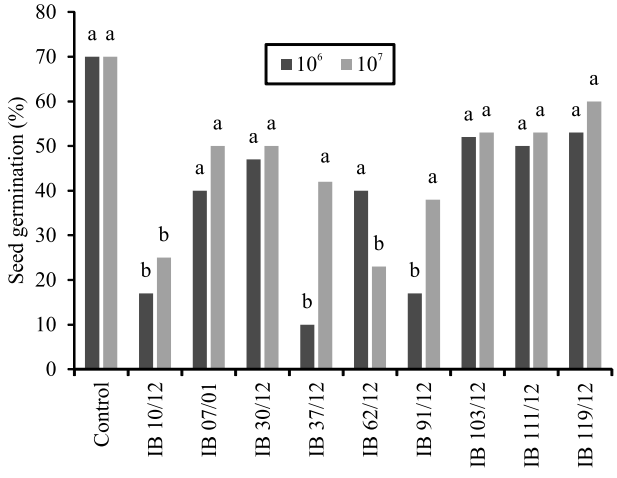

Figure 1. Effect of strains of *Trichoderma* spp. on 'Pintado' soybean (*Glycine max*) seed germination in vitro (Petri dishes). Seeds were treated with conidial suspensions at concentrations of 10^6 and 10^7 per mL. In the control, seeds were treated with autoclaved water. Values are presented as percentage of germinated seeds (n=20). Data presented are the means of 3 repetitions. Values indicated by equal letters do not differ significantly by the Scott-Knott test, at 5% probability.

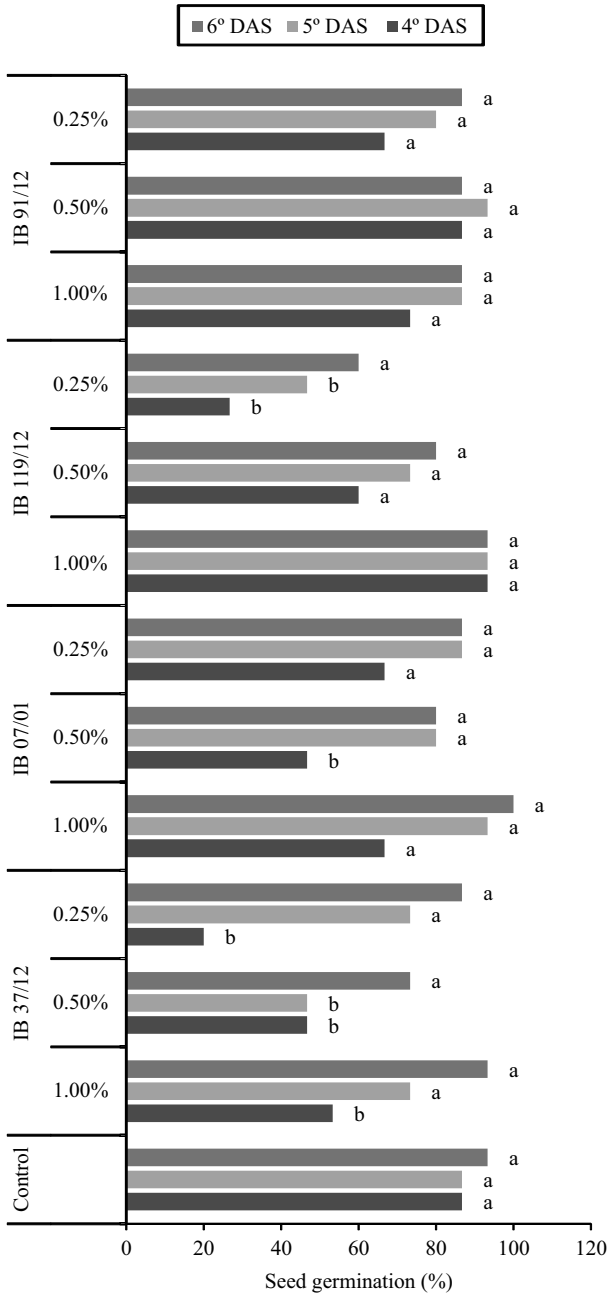

Figure 2. Effect of strains of *Trichoderma* spp. on 'Pintado' soybean (*Glycine max*) seed germination in vivo (pots with substrate). Strains of *Trichoderma* spp. were grown on autoclaved rice grains and incorporated to the substrate at the concentrations of 0.25, 0.5, and 1.0% (weight/volume). The control treatment consisted of autoclaved rice grains without *Trichoderma* spp. Values are presented as percentage of germinated seeds (n=3). Data presented are the means of 5 repetitions. Values indicated by equal letters do not differ significantly by the Scott-Knott test, at 5% probability. DAS, days after sowing.

L.) seed germination but also enhance the growth of the plant. According to the authors, the results are highly depend on strain, method of inoculum application and preparation.

In the biological control assay performed in the greenhouse, typical white mold symptoms could not be observed on the inoculated plants; however, the presence of strains of *Trichoderma* spp. on the substrate increased seed germination in the presence of the pathogen in at least one of the experimental replicates (Table 1). Differences between treatments could not be observed in the first experiment. Five strains reduced seed germination inhibition by the pathogen in the second experiment and all strains had this effect in the third experiment. Strains IB 07/01 and IB 37/12 had the best performance when the last two experiments were considered. Six strains enhanced seed germination even in the absence of the pathogen in the third experiment. Tančić et al. (2013) also found improvement in soybean seed germination, emergence, and vigor subjected to treatment with *Trichoderma* spp. Brotman et al. (2013) reported increase in the emergence and dry matter of *Arabidopsis thaliana* and cucumber grown on soil amended with *Trichoderma* spp. The results obtained corroborate those of Benítez et al. (1998), who showed that *Trichoderma* spp. synthesize growth factors that increase seed germination rate. The absence of effect of strains of *Trichoderma* spp. in the first experiment can be explained by the low temperatures (average 19.1ºC) observed in June 2013. Several abiotic factors, such as temperature and humidity, can affect the activity of *Trichoderma* spp., which are better adapted to temperatures between 25 and 30ºC (Bomfim et al., 2010; Akrami et al., 2011).

Most strains of *Trichoderma* spp. promoted an increase in above-ground dry matter in the three biological control experiments in the presence of the pathogen (Table 2). The highest levels were observed for strains IB 103/12 (56%), IB 37/12 (33%), and IB 07/01 (30%) in the first experiment; IB 07/01 (61%), IB 10/12 (28%), and IB 62/12 (27%) in the second experiment; and IB 62/12 (67%), IB 91/12 (63%), and IB 111/12 (53%) in the third experiment. Therefore, soybean plants had better development on substrate infected with *S. sclerotiorum* when strains of *Trichoderma* spp.

Table 1. 'Pintado' soybean (*Glycine max*) germinated seeds in substrate infested with *Sclerotinia sclerotiorum* in the presence or absence of strains of *Trichoderma* spp.[1].

Treatment	First assay June 2013	Second assay December 2013	Third assay January 2014
Infected control[2]	4.2a	2.6b	3.0c
Control[3]	4.2a	4.4a	4.2b
IB 10/12	4.4a	4.6a	4.0b
IB 07/01	4.4a	5.2a	5.0a
IB 30/12	4.2a	3.4b	4.8a
IB 37/12	4.6a	4.8a	4.7a
IB 62/12	4.4a	3.2b	4.5a
IB 91/12	4.4a	3.6b	4.8a
IB 103/12	4.6a	3.4b	4.7a
IB 111/12	4.2a	4.8a	4.3b
IB 119/12	4.4a	4.2a	4.2b
CV (%)	13.65	25.22	12.17

[1]Means followed by equal letters, in the columns do not differ significantly by the Scott-Knott test, at 5% probability. [2]Infected control, plants inoculated with *S. sclerotiorum*. [3]Control, plants not inoculated with either pathogen or strains of *Trichoderma* spp. CV, coefficient of variation.

Table 2. Above-ground dry matter weight (DMW) of 'Pintado' soybean (*Glycine max*) grown on substrate infested with *Sclerotinia sclerotiorum* in the presence or absence of strains of *Trichoderma* spp.[1].

Treatment	First assay June 2013 DMW (g)	Second assay December 2013 DMW (g)	Third assay January 2014 DMW (g)
Infected control[2]	0.877±0.04d	0.8780±0.026c	0.5156±0.012d
Control[3]	1.051±0.07c	0.7239±0.056d	0.7657±0.027b
IB 10/12	0.901±0.07d	1.1306±0.033b	0.6423±0.029c
IB 07/01	1.143±0.03b	1.4202±0.040a	0.7076±0.028b
IB 30/12	1.068±0.01c	0.9533±0.092c	0.7361±0.049b
IB 37/12	1.170±0.03b	1.0112±0.062c	0.7585±0.052b
IB 62/12	0.997±0.03c	1.1219±0.066b	0.8633±0.028a
IB 91/12	0.986±0.02c	0.8985±0.037c	0.8423±0.024a
IB 103/12	1.369±0.09a	0.7085±0.043d	0.7527±0.035b
IB 111/12	1.035±0.05c	0.8564±0.081c	0.7909±0.034b
IB 119/12	0.821±0.04d	0.8270±0.066c	0.6518±0.024c
CV (%)	11.3	13.6	10.2

[1]Means followed by equal letters, in the columns do not differ significantly by the Scott-Knott test, at 5% probability. [2]Infected control, plants inoculated with *S. sclerotiorum*. [3]Control, plants not inoculated with either pathogen or strains of *Trichoderma* spp. CV, coefficient of variation.

were present, showing the potential of these strains to reduce deleterious effects of the pathogen. Similar results were obtained by Ojaghian (2011), who showed that several species of *Trichoderma* reduced disease severity caused by *S. sclerotiorum* on potato (*Solanum tuberosum* L.) in a greenhouse experiment and also reduced disease incidence in field conditions.

Sclerotinia sclerotiorum can cause an average reduction of 46% in above-ground dry matter of soybean plants when seeds are inoculated with the pathogen (Botelho et al., 2013). In the conditions of the present study, reductions of up to 32.6% were observed (Table 3). However, positive effect of *Trichoderma* spp. on the growth of soybean plants in the presence of *S. sclerotiorum* has been reported by Guareschi et al. (2012), who found an increase in root system dry matter of up to 44.9% 60 days after emergence. Therefore are reports of growth promotion by *Trichoderma* spp. in the absence of pathogens for other plant species such as tomato (*Solanum lycopersicum* L.) (Fontenelle et al., 2011), common bean (*Phaseolus vulgaris* L.) (Pedro et al., 2012), cucumber (*Cucumis sativus* L.) (Silva et al., 2011), and *Arabidopsis* sp. (Korolev et al., 2008). This beneficial effect has been attributed to many factors such as protection against rhizosphere pathogens, hormone production, increase of growth and development of the root system, improved absorption and translocation of mineral nutrients and increase of solubility and availability of micronutrients (Contreras-Cornejo et al., 2009).

In the greenhouse experiments, white mold symptoms on inoculated soybean plants could not be observed. Ideal conditions for white mold occurrence are high humidity, temperatures between 15 and 25ºC, and low light incidence (Fischer et al., 2014). The lack of one of these factors can delay or interrupt disease progress. During our experiments, maximum average temperatures were 22.6ºC in June 2013, 28.5ºC in December 2013, and 31.7ºC in January 2014, with relative humidity around 50% (Figure 3). These conditions may not have favored symptom manifestation induced by the pathogen. Likewise, Guareschi et al. (2012) did not observe disease symptoms on the soybean plants inoculated with *S. sclerotiorum* due to the high temperatures (above 26ºC) during the experimental period.

At the end of the biological control experiments, it was confirmed that all strains of *Trichoderma* spp. were able to colonize the root system of soybean plants. This is considered an important feature for *Trichoderma* to act as a biological control agent or to promote plant growth since it allows competing for space or nutrients with soil pathogens (Samuels,

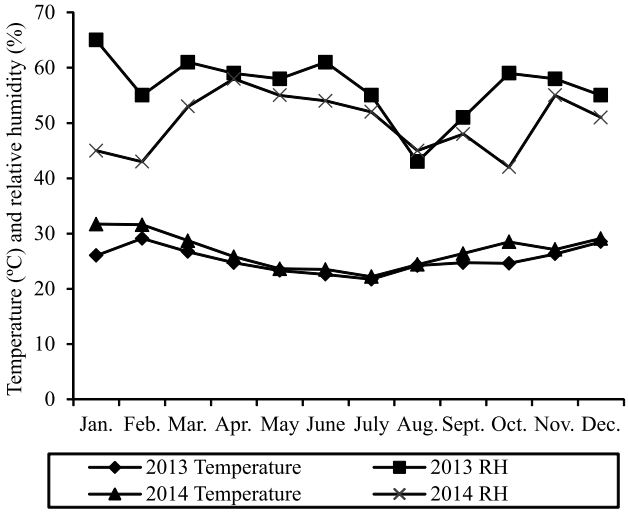

Figure 3. Monthly average temperatures and relative humidity in the municipality of São Paulo, in 2013 and 2014, according to IAG/USP (Boletim…, 2015).

Table 3. Relative growth of 'Pintado' soybean (*Glycine max*) on substrate infested with *Sclerotinia sclerotiorum* in the presence or absence of strains of *Trichoderma* spp. based on above-ground dry matter weight.

Treatment	Growth (%)		Growth (%)		Growth (%)	
	*	**	*	**	*	**
Infected control[(1)]	0.00	-16.56	0.00	21.27	0.00	-32.67
Control[(2)]	19.83	0.00	-17.54	0.00	48.26	0.00
IB 10/12	2.75	-14.23	28.77	56.16	24.47	-16.12
IB 07/01	30.26	8.73	61.76	96.17	37.13	-7.59
IB 30/12	21.77	1.65	8.58	31.68	42.66	-3.87
IB 37/12	33.40	11.36	15.18	39.68	46.99	-0.95
IB 62/12	13.63	-5.14	27.78	54.96	67.31	12.75
IB 91/12	12.36	-6.21	2.34	24.11	63.24	10.00
IB 103/12	56.06	30.27	-19.30	-2.14	45.88	-1.70
IB 111/12	17.97	-1.52	-2.45	18.30	53.28	3.29
IB 119/12	-6.38	-21.85	-5.80	14.24	26.32	-14.88

[(1)]Infected control, plants inoculated with *S. sclerotiorum*. [(2)]Control, plants not inoculated with either pathogen or strains of *Trichoderma* spp. *Calculated by comparison with infected control plants. **Calculated by comparison with control plants.

2006). Furthermore, colonization allows an intimate and complex interaction with the plant through direct contact and internal colonization of root tissues (Hermosa et al., 2012).

The phylogenetic tree constructed with the TEF sequences of the strains of *Trichoderma* spp. of the present study and sequences retrieved from the GenBank allowed species identification (Figure 4). Strains IB 62/12, IB 103/12, and IB 111/12 belong to *T. koningiopsis*; strains IB 10/12, IB 37/12, and IB 91/12 belong to *T. asperelloides*; strains IB 07/01 and IB 30/12 belong to *T. atroviride*; and strain IB 119/12 belongs to *T. virens*. These species have already been reported as biocontrol agents for *S. sclerotiorum* (Ethur et al., 2005; Matroudi et al., 2009; Ojaghian, 2011; Lopes et al., 2012).

The obtained results show the potential of strains of *Trichoderma* spp. for the biological control of white mold of soybeans. Further research in field conditions are required to select the most suitable strains for commercial product formulation and registration.

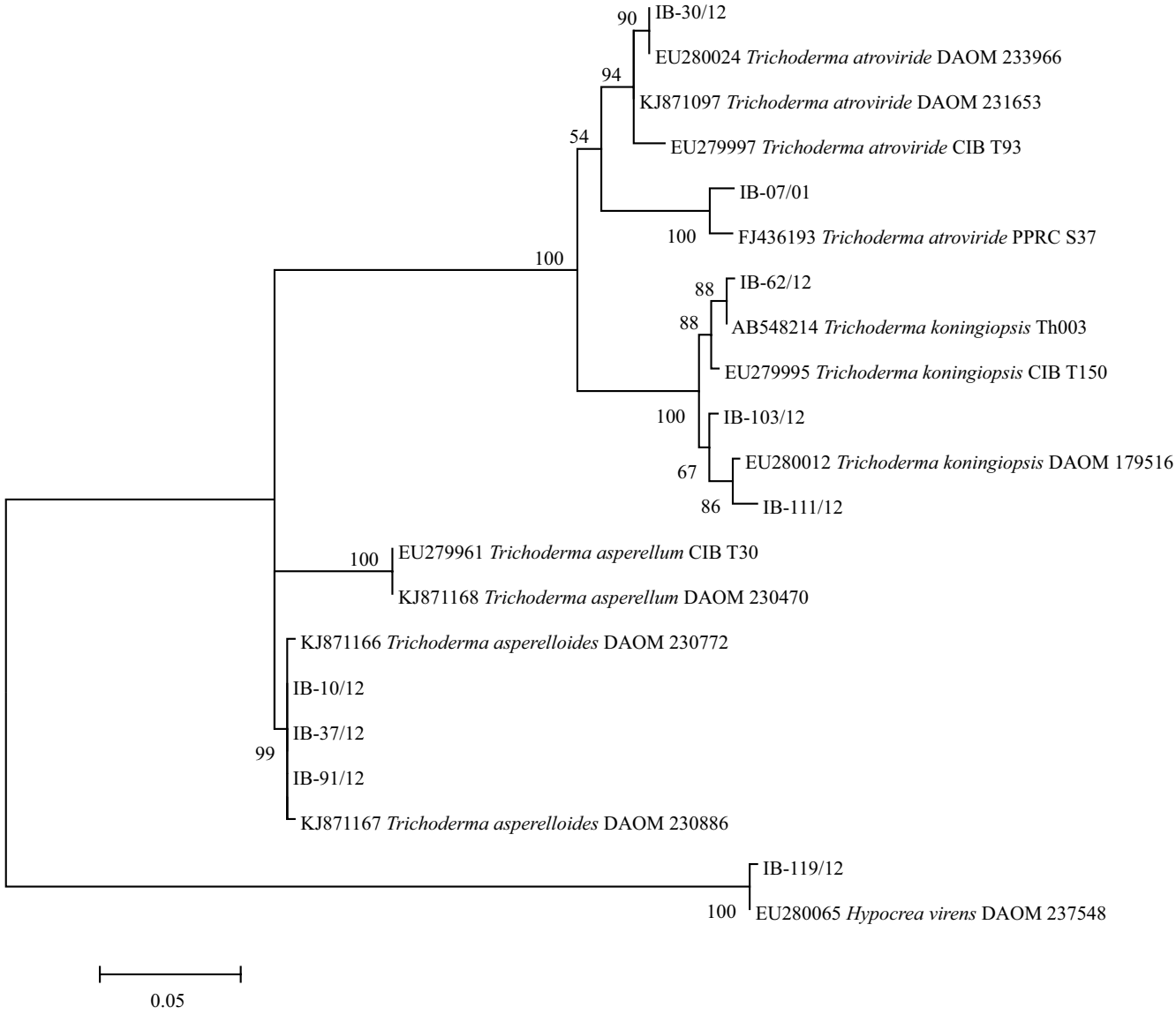

Figure 4. Phylogenetic tree constructed by the maximum likelihood method based on Kimura 2-parameter model with sequences of the translation elongation factor (TEF) from strains of the present study and sequences retrieved from the GenBank. Values next to branches show bootstrap values in percentage for 1000 repetitions.

Conclusions

1. Among 120 strains of *Trichoderma* spp., 22 resulted in 100% inhibition of *Sclerotinia sclerotiorum* sclerotia germination in vitro.

2. Nine strains showed the ability to protect soybean (*Glycine max*) seed germination and plant growth from deleterious effect of the pathogen in at least one experiment.

3. The best performing strains were identified as *T. koningiopsis*, *T. asperelloides*, *T. atroviride*, and *T. virens*.

Acknowledgments

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