

TRANSMISSION OF *Sclerotinia sclerotiorum* FROM SOYBEAN SEED TO SEEDLINGS

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ABSTRACT

This paper evaluates the transmission of *S. sclerotium* from infected soybean seeds to seedlings. Results showed that a lower emergence percentage and speed was obtained after 24h inoculation without asepsis; the transmission of *S. sclerotiorum* therefore occurred from soybean seeds to symptomatic seedlings. Asymptomatic seedlings do not present the fungus, indicating no transmission.

Keywords: Agar-bromophenol medium, Emergence percentage, *Glycine max*, Health test

TRANSMISSÃO DE *Sclerotinia sclerotiorum* DE SEMENTES PARA PLÂNTULAS DE SOJA**RESUMO**

Essa pesquisa avaliou a transmissão de *S. sclerotiorum* de sementes de soja infectadas para plântulas. Os resultados mostraram baixa porcentagem de emergência para as sementes que foram inoculadas por 24h sem assepsia; a transmissão de *S. sclerotiorum* ocorre de sementes de soja para plântulas com sintomas. Plântulas assintomáticas não apresentam fungo, indicando que não há transmissão.

Palavras-chave: Meio ágar-bromofenol, Porcentagem de emergência, *Glycine max*, Teste de sanidade

INTRODUCTION

Sclerotinia sclerotiorum (cottony white rot) is a cosmopolitan fungus that infects more than 500 species of plants worldwide (SAHARAN & MEHTA, 2008).

The control of this pathogen in several crops has been difficult due to its capacity to produce resistance structures (sclerotia), which could survive in the soil for several years, even in adverse conditions. Infected plants show the presence of fluffy white

mycelium in lesions (BOLTON et al., 2006). In soybean (*Glycine max* (L.) Merrill), the symptoms appear mainly during the reproductive stage (KIMATI et al., 2005), generally in the third middle of the plants, reaching the main stem, petioles, leaves and pods (BOLTON et al., 2006).

The seed is the main dissemination vehicle, and the dissemination might be by the presence of sclerotia among seeds (HENNEBERG et al., 2012; KIMATI et al., 2005). Dormant mycelia have been shown to remain viable for more than three years, in infected seeds, without appreciable decrease in viability, when the air-dried seeds were stored at 4 °C (TU, 1988). The same author showed that when the seeds were sown, many of them did not germinate and were rotted by the fungus and there was the formation of many sclerotia. These sclerotia were then able to germinate both mycelially and carpogenically, depending on environmental conditions.

Rotation with susceptible crops, susceptible cultivars, infected soils and usage of infected seeds are factors which might be involved in the increase of the occurrence and the severity of *S. sclerotiorum* in the production fields. Therefore, one of the ways to preventing *S. sclerotium* from entering in free areas is the

use of healthy or adequately treated seeds (KIMATI et al., 2005).

This pathogen has therefore been important in quality control programmes for seed companies, since they can be important dissemination vehicles of *S. sclerotiorum*, through sclerotia mixed in the seed mass or mycelia present in internal tissues (AGRIOS, 2005). However, the transmission to the seedlings is not completely understood. Experiments performed with common beans (KOCH, 2001) naturally infected by *S. sclerotiorum* did not produce seedlings with white mould symptoms.

This paper aimed to investigate the transmission of *S. sclerotium* from infected soybean seeds to seedlings.

MATERIAL AND METHODS

Two experiments were performed, in two consecutive years, using soybean seeds from the cultivar TMG123-RR, produced in Alto Garças area (16°56'38''S and 53°31'41''W), Mato Grosso State, Brazil in 2009/2010 season, which were, initially characterized in terms of their health and germination.

For the experiments the seeds were inoculated with *S. sclerotiorum* to a level of incidence that could be studied. Generally,

the percentage of naturally infected seeds is, too low, varying from 0.25% to 1%.

All the seeds were initially submitted to superficial asepsis with sodium hypochlorite 1% for three minutes, and kept in the laboratory at room temperature for 24 hours. Subsequently, the seeds were placed in Petri dishes containing pure pathogen colonies, (characterized by white and fluffy mycelia), and were kept in an incubator at 20 ± 2 °C for 18h and 24h. The same *S. sclerotiorum* isolate was used in the two consecutive years experiments. Simultaneously some non-inoculated seeds were put in direct contact with the PDA medium (potato-dextrose-agar) without the fungus, for the same 18h or 24h for later evaluation of the possible effects of this procedure on the seedlings emergence. After this inoculation phase, the seeds were taken out of the Petri dishes and kept in the laboratory at room temperature for 24h again, so that the seed moisture content achieved hygroscopic equilibrium with the relative humidity of the air.

The incidence of *S. sclerotium* was evaluated using agar-bromophenol medium (PDA+105 mg.L⁻¹ of bromophenol blue, 150 mg.L⁻¹ of streptomycin sulphate and 140 mg.L⁻¹ of penicillin) (STEADMAN et al., 1994) and it was assessed for four

treatments: seed inoculation for 18h or 24h with asepsis in 1% sodium hypochlorite for three minutes, and inoculation for 18h or 24h without asepsis. For each treatment, four replicates of 50 seeds were incubated at 20 ± 2 °C with alternation (12h) of light near ultraviolet (NUV). They were observed daily to check the yellowish halos around the seeds. The presence of yellowish halo is an indication of the presence of the *S. sclerotiorum*, because the oxalic acid produced by the fungus changes the colour of the medium from blue to yellow. In order to confirm the presence of *S. sclerotium*, the dishes were observed under white light, which made it possible to visualize any mycelium coming from the seeds on the culture medium in the area of the yellowish halo. The result of incidence of infection was expressed as percentage of seeds with the presence of *S. sclerotiorum*. The test used to evaluate the transmission of *S. sclerotiorum* from the seeds to the seedlings was the emergence test in greenhouse. The physiological potential of the seeds was also evaluated through speed and percentage of the seedling emergence. For this, three more treatments were added to the four already described: control seeds (not submitted to inoculation processes) and seeds which were

in contact with the PDA medium without the fungus for 18h and 24h

Four replicates of 50 seeds of each treatment were sown in trays containing vermiculite and kept in a greenhouse until seedlings developed, for at least 20 days. The variations in greenhouse temperature were registered (Figure 1). During development, the speed of emergence index (MAGUIRE, 1962) and the emergence percentage were evaluated. Emerged seedlings were considered as those in which the cotyledons were completely grown out of the substrate, angled in parallel to its surface.

At the final emergence stage, seedlings and seeds were assessed for the presence of mycelium and seedlings which had died after emergence due to the presence of *S. sclerotiorum*. Some seeds with mycelium could be seen on the surface of the

substrate. In addition, the transmission rate of *S. sclerotiorum* to soybean seedlings was determined based on the division of the percentage of seedlings with mycelium by the percentage of pathogen incidence in the health test.

Twenty days after the sowing, 20 emerged seedlings were randomly chosen with or without symptoms of white mould. These seedlings were cut into two segments of 1cm; one segment was taken from the epicotyl and the other from the hypocotyl. This tissue was submitted to asepsis with 1% sodium hypochlorite for 1min and subsequently alcohol 70% for 2s, placed in agar-bromophenol medium and incubated in an incubation room at 20 ± 2 °C with NUV alternation (12h) in order to confirm the transmission.

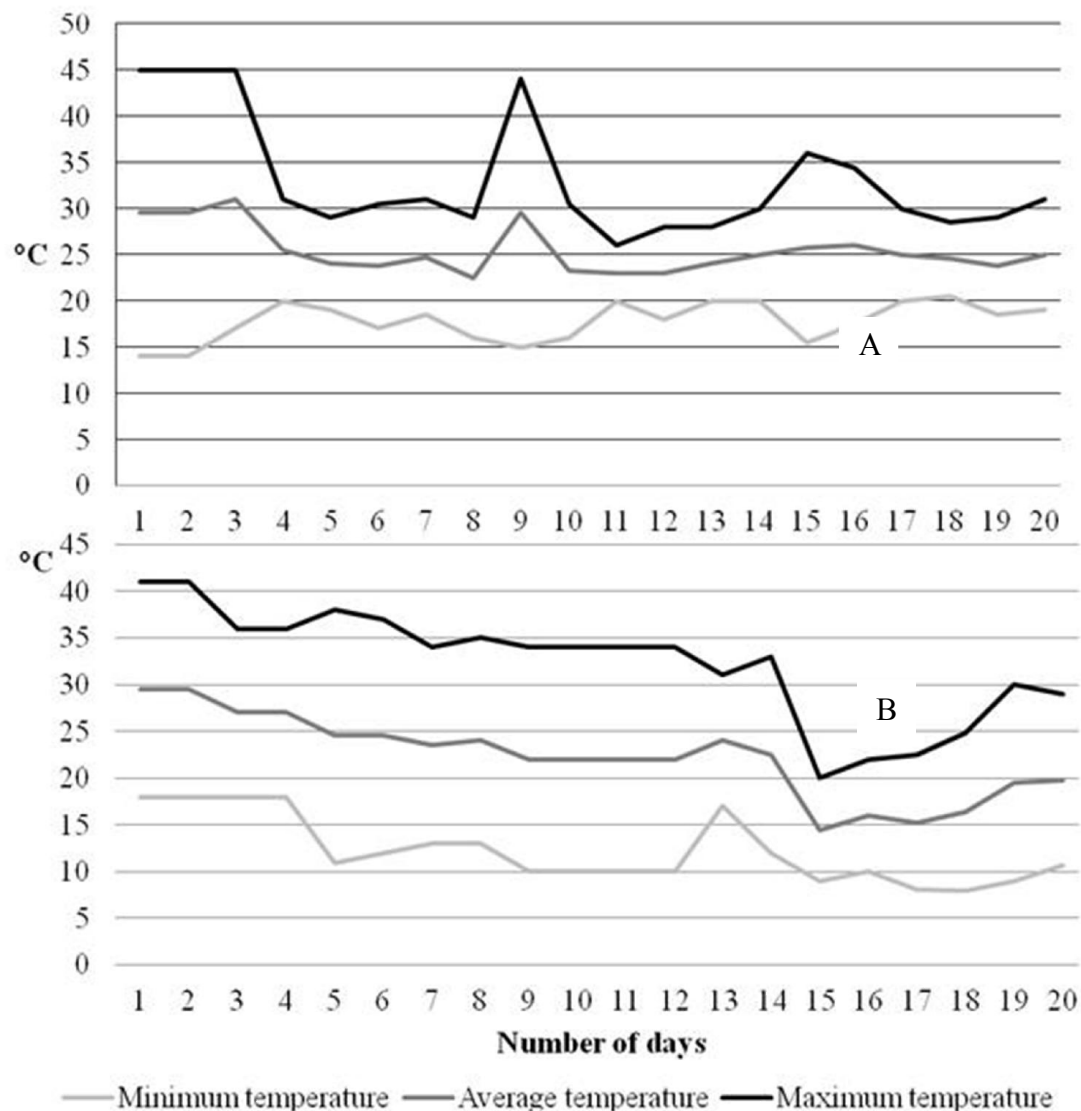


Figure 1. Greenhouse maximum, average and minimum temperature, in °C, during the period of the seedling emergence test of soybean seeds in the first (A) and second (B) experiment (Piracicaba, A Oct 2010/ B May 2011)

Along with that analysis, parts of the tissue of the seedlings, such as cotyledons and hypocotyls, in which the symptoms of *S. sclerotiorum* were unclear, were put in contact with the agar-bromophenol medium in order to confirm the presence of the pathogen. Not only the segments of hypocotyls, but also other tissues of

seedlings were individually identified on the Petri dishes to facilitate assessments.

The experimental design was completely randomized with four treatments and four replications (for the experiment of *S. sclerotium* detection in the seeds) and seven treatments and four replications (for the experiment of transmission). The average

of each treatment was compared by the Tukey test at 5% of probability.

RESULTS AND DISCUSSION

The initial germination of the control seeds used for this research was 92% and the incidence of *S. sclerotiorum* was 0%, which demonstrates the importance of the inoculation procedure. The early determination of the seed health was important for the decision made regarding the inoculation later carried out, because there was not natural incidence of the pathogen.

In the seed health test through agar-bromophenol medium, a higher incidence of the pathogen was observed in the seeds inoculated for 24h compared to those inoculated for 18h in the treatments with and without asepsis (Table 1 and Figure 2). The incidence of the fungus was higher in the treatments without asepsis. However, the asepsis with sodium hypochlorite allowed estimating the level of infection of *S. sclerotiorum* in the seeds. The contact of those with sodium hypochlorite at 1% for three minutes caused the death of the fungi that were on the surface of the seeds, which left, at

the end of the health test, only the mycelium which was present inside the seeds. The inoculum that remained after asepsis may have lower capacity of survival for longer time and have a lower probability of causing disease in the plant (BOTELHO et al., 2008).

Besides having shown good results for the incidence evaluation, the agar-bromophenol medium, used for the health test, needs to be kept and evaluated until the moment of mycelium and/or sclerotia formation for the confirmation of the fungus. In many cases, it was observed the presence of yellowish halo around the seeds; however, the occurrence of other contaminating fungi, also acidify the agar-bromophenol medium and make it yellow, like fungi of the genera *Aspergillus* and *Rhizopus*, among others (HENNEBERG et al., 2012).

The percentage of emerged seedlings was lower for all the inoculation treatment with *S. sclerotiorum* on the first experiment, while in the second, reduction occurred only on the seeds which were in contact with the fungus for 24h and was not submitted to asepsis (Table 1), demonstrating a better efficiency of the first inoculation.

Table 1. *Sclerotinia sclerotiorum* incidence (%) evaluated by health test in agar-bromophenol medium (H), emerged plants (EP) and speed emergence index (SEI) for inoculation treatments in soybean seeds, in both experimental periods (Piracicaba, First Oct 2010/ Second May 2011).

FIRST EXPERIMENT			
Treatment	H	EP	SEI
	%		
Control	-	93 a	8.5 a
18 h PDA ¹	-	89 a	7.3 a
24 h PDA ²	-	73 b	5.3 b
18 h a. ³	16 a	76 b	5.9 b
24 h a. ⁴	36 b	72 b	5.8 b
18 h n.a. ⁵	52 c	64 b	5.2 b
24 h n.a. ⁶	62 c	42 c	3.6 c
CV (%)	3.9	7.4	8.5
SECOND EXPERIMENT			
Treatment	H	EP	SEI
	%		
Control	-	97 a	8.4 a
18 h PDA	-	95 a	7.7 ab
24 h PDA	-	95 a	8.6 a
18 h a.	4 a	90 a	7.8 ab
24 h a.	9 b	87 a	7.4 ab
18 h n.a.	32 c	88 a	7.9 ab
24 h n.a.	49 c	70 b	6.1 b
CV (%)	12.3	1.2	5.8

¹ 18 hours in contact with PDA

² 24 hours in contact with PDA

³ 18 hours of inoculation with *S. sclerotiorum* with asepsis

⁴ 24 hours of inoculation with *S. sclerotiorum* with asepsis

⁵ 18 hours of inoculation with *S. sclerotiorum* without asepsis

⁶ 24 hours of inoculation with *S. sclerotiorum* without asepsis

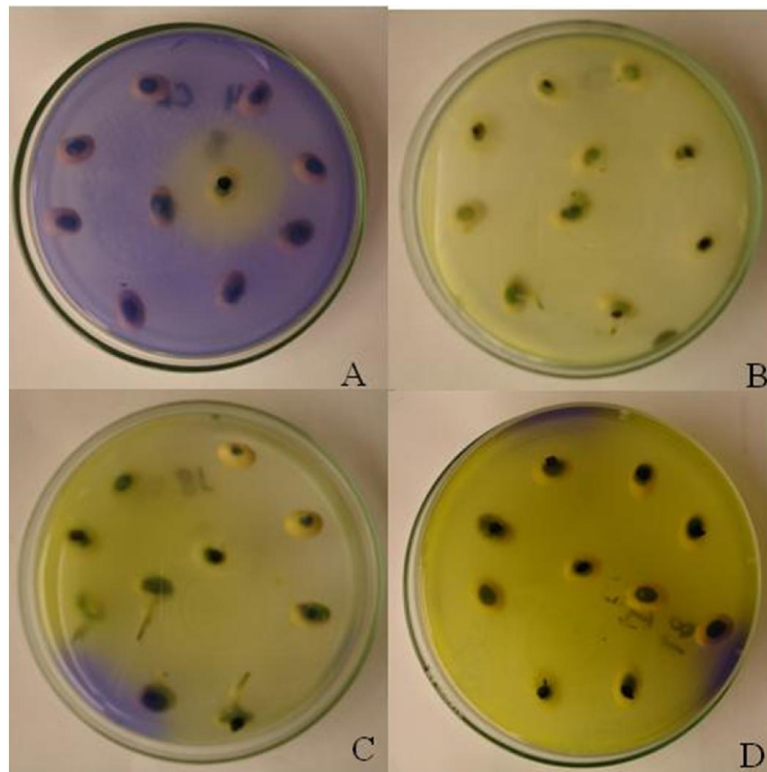


Figure 2. Evaluation of *Sclerotinia sclerotiorum* incidence on agar-bromophenol medium of inoculated soybean seeds. A - inoculation for 18 hours with asepsis; B - inoculation for 18 hours without asepsis; C - inoculation for 24 hours with asepsis; D - inoculation for 24 hours without asepsis (Piracicaba, Oct 2010/May2011).

The average greenhouse temperature for both experiments was similar during the whole period (Figure 1), which shows that the differences in percentage of emerged seedlings were probably due to the inoculation process and pathogen efficiency and not to the temperature in the greenhouse. In the second experiment, the effect of the inoculation was less efficient than in the first, probably because of successive pricking-outs for the maintenance of the isolate. The incidence of the pathogen was lower, but it also decreased the seedlings emergence and the emergence speed. The asepsis reduced the incidence of

the pathogen and, as a consequence, reduced its effect on the seedlings emergence.

The inoculation for 18h did not interfere in the physiological potential of the seeds; therefore, the reduction of the emergence of this treatment, for the first experiment, might be exclusively due to the effect of the pathogen. Hoffman et al. (1998) reported that, for the soybean cultivars P9381, Yale, P9342 and A3304, the seed germination was reduced with the increase of incidence of *S. sclerotiorum*. The same author observed transmission of the fungus

in the maximum 0.7% for seedlings in germination roll.

Based on the seedlings emergence data in greenhouse, it was observed that the contact of 24h in the medium culture without the fungus affected the physiological potential of the seeds for the first experiment (Table 1), which caused reduction of 20 percentage points in the seedling emergence. The contact of the seeds with the medium culture decreased the physiological potential of the seeds for the first experiment. In this case, the PDA medium could be less consistent or could have condensed water over the medium. Both conditions would allow a higher absorption of water by the seeds and, as a consequence, cause a reduction of the physiological potential. Moreover, the contact with the medium might have decreased the seeds vigor; in addition, the temperature conditions and relative humidity of the air and of the substrate in the greenhouse could have contributed for the lowering of the percentage and speed of seedlings emergence in the first experiment.

The presence of *S. sclerotiorum* in the seeds also interfered in the speed of the seedlings emergence similarly to the percentage of emergence for both experiments. In the treatment with seeds

inoculated for 24h without asepsis, the speed emergence index (SEI) was lower as compared to the other treatments in the first experiment (Table 1).

The percentage of dead seedlings (DSe) and seed (DS), seedlings (SeM) and seeds with mycelium (SM) and the transmission rate (TR), for both experiments, are in Table 2. Dead seedlings were observed only on treatments inoculated with the fungus for the first experiment and all of them there were *S. sclerotiorum* mycelial growth.

For the seeds submitted to contact with the fungus for 18h at the first experiment, there was a difference in the number of dead seeds between the ones of direct contact with PDA only and the inoculated, submitted or not to asepsis. These results showed that after inoculation with the fungus, it set itself in the seeds, which caused their deaths. Among the seeds submitted only to PDA and those inoculated for 24h with asepsis, there was not a difference in the percentage of dead seeds, despite the presence of 5% of seeds with mycelium of *S. sclerotiorum*. For the other analysis, the highest and the lowest transmission rate was of 6.3% and 3.2%, respectively, for the treatments of seeds

inoculated for 18h with asepsis and seeds inoculated for 24h without asepsis (Table 2).

In the second experiment, mycelium was observed in all cases of dead seed, which shows that these deaths were caused by the presence of the pathogen and not by the inoculation procedure, because when the seeds stayed only in the PDA medium for 18h and 24h, there was no occurrence of dead seeds (Table 2). Only for the inoculation treatment for 24h without asepsis the presence of seedling with mycelium was observed, that resulted in a transmission rate of 4.1%.

Similarly to the seedlings development evaluations, when the occurrence of dead seeds or seedlings with mycelium was evaluated, the highest incidences occurred in the first experiment. Therefore, a transmission rate of *S. sclerotiorum* was observed in soybean seedlings, being 5.5% and 3.2% for the seedlings from seeds submitted to 24h of inoculation with and without asepsis, respectively, and 6.3% and 5.8% for the ones inoculated for 18h with and without asepsis, respectively, for the first experiment (Table 2). For the second experiment a transmission rate of 4.1% was observed for the seedlings from seed submitted to inoculation of 24h without asepsis. These high transmission

rates showed that, when associated to the seed, the transmission of *S. sclerotiorum* to seedlings is very important and may be a determining factor in the health quality of a soybean field.

Thereby, besides the low transmission rate in symptomatic seedlings, if we consider a population of 240 thousand plants per hectare and considering the percentage of seedlings with a 2% mycelium, it would be possible to obtain 4,800 seedlings with incidence of the pathogen which may or not result in infected plants with the formation of sclerotia. If it is considered that up to 5% of the seed died due to the presence of the pathogen, in the same population of 240 thousand plants per hectare there would be more 12,000 source of inoculum in the field.

In many dead seedlings, it was observed symptoms of the disease and mycelium of the fungus in the strap of the hypocotyls (Figure 3C), in the cotyledons (Figure 3A, 3B, 3C, 3E, 3F) and in the seedcoat (Figure 3D), confirmed by the incubation of these tissues in agar-bromophenol medium. These symptoms were characterized, at first, as “soaked” brown colored tissues with later growth of mycelium in the surface.

Table 2. Percentage of dead seedlings (DSe), dead seeds (DS), seedlings with mycelium (SeM), seeds with mycelium (SM) and transmission rate (TR) of soybean seeds inoculated with *S. sclerotiorum* in both experimental periods. (Piracicaba, First Oct 2010/Second May2011)

FIRST EXPERIMENT					
Treatment	DSe	DS	SeM	SM	TR
	%				
Control	0 a	8 a	0 a	0 a	-
18 h PDA ¹	0 a	11 a	0 a	0 a	-
24 h PDA ²	0 a	27 b	0 a	0 a	-
18 h a. ³	1 a	25 b	1 a	1 a	6.3
24 h a. ⁴	2 a	28 b	2 a	5 b	5.5
18 h n.a. ⁵	3 a	36 b	3 a	4 b	5.8
24 h n.a. ⁶	2 a	58 c	2 a	5 b	3.2
CV (%)	98.0	15.3	98.0	73.5	
SECOND EXPERIMENT					
Treatment	DSe	DS	SeM	SM	TR
	%				
Control	0	0 a	0 a	0 a	-
18 h PDA	0	0 a	0 a	0 a	-
24 h PDA	0	0 a	0 a	0 a	-
18 h a.	0	1 a	0 a	0 a	0.0
24 h a.	0	2 a	0 a	2 ab	0.0
18 h n.a.	0	3 ab	0 a	3 b	0.0
24 h n.a.	0	5 b	2 a	5 b	4.1
CV (%)		49.3	96.3	18.6	

¹ 18 hours in contact with PDA

² 24 hours in contact with PDA

³ 18 hours of inoculation with *S. sclerotiorum* with asepsis

⁴ 24 hours of inoculation with *S. sclerotiorum* with asepsis

⁵ 18 hours of inoculation with *S. sclerotiorum* without asepsis

⁶ 24 hours of inoculation with *S. sclerotiorum* without asepsis



Figure 3. Soybean seedlings with *Sclerotinia sclerotiorum* symptoms. A, B, E and F: symptoms in cotyledons, C: symptom in strap hypocotyl, D: mycelium on the surface of cotyledons (Piracicaba, Oct 2010/May2011).

Bolton et al (2006), in his research about *Sclerotinia sclerotiorum* biology, affirmed that this is a necrotrophic homothallic pathogen. As *S. sclerotiorum* has a large host range, there are no unique symptoms that belong to all plants infected

by this fungus. However, lesions usually develop into necrotic tissues that subsequently develop patches of fluffy white mycelium, which are the most obvious sign of plants infected with *S. sclerotiorum* (BOLTON et al., 2006).

The symptoms in seedlings and seeds incidences were due to the production of oxalic acid by *S. sclerotiorum* (KIM et al., 2008), the same acid which alters the color of the agar-bromophenol medium. Livingstone et al. (2005) applied varying concentrations of oxalic acid to the surface of detached peanut leaflets that showed that the higher oxalic acid concentration, the greater the lesions on leaflets. Oxalic acid may aid in infection through a number of proposed routes, including acidification to facilitate cell wall-degrading enzyme activity, through pH-mediated tissue damage, or via sequestration of Ca^{2+} ions (LIVINGSTONE et al. 2005). The oxalate secreted by the fungus induces the production of reactive oxygen species in the plant host that appears to trigger a programmed cell death pathway, which results in plant cell death and the generation of a suitable environmental niche for fungal pathogenic development, nutrients acquisition and the establishment of a necrotrophic relationship (KIM et al., 2008).

In some seedlings there was mycelial growth in the cotyledons and in the seedcoat (Figure 3D), which prevented their opening and the exposition of the primary leaves. After the mycelium growth, the tissues underwent dehydration, which caused death

of the seedling. However, in some cases, the presence of symptoms was observed in the cotyledons without the occurrence of abnormality or seedlings death, once the damage was lower than 50% of the area of the cotyledons and it was limited to the boundaries of this tissue.

On the test performed with asymptomatic seedlings, 20 days after sowing, the presence of *S. sclerotiorum* was not observed either in the epicotyls or in the hypocotyls. The transmission was verified only through the observation of symptomatic seedlings and the confirmation from the incubation in agar-bromophenol medium. When the symptoms occurred in the cotyledons, the presence of the fungus was not observed in the hypocotyls and epicotyls, as it could be seen in beans (KOCH, 2001).

CONCLUSION

As a conclusion, the transmission of *S. sclerotiorum* occurs from soybean seeds to symptomatic seedlings. Asymptomatic seedlings do not present the fungus, which indicates no transmission.

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