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FUNGICIDES AND BIOCONTROL AGENTS IN REDUCING CARPOGENIC GERMINATION OF SCLERODIA *SCLEROTINIA SCLEROTIORUM IN VITRO*

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Abstract: The experiment in the laboratory was conducted in 2020, where sclerotia of *Sclerotinia sclerotiorum* were subjected to the application of different chemical and biological fungicides, described as follows: 1- Methyl thiophanate + Fluazinam (37.5+37.5%); 2- Fluazinam (50%); 3- Dimoxystrobin +Boscalid (20+20%); 4- Methyl thiophanate (87.5%); 5- Bixafen + Prothioconazole + Trifloxyxtrobin (12.5+17.5+15.0%); 6- Procymidone (50%); 7- Carbendazim (50%); 8- *Trichoderma harzianum* 1306 (4,8%); 9- *Trichoderma harzianum* cepa T-22 (1%); 10- *Bacillus subtilus* linh. QST 713. Doses indicated by the manufacturers in volume of syrup were used. 100 L. ha^{-1}. The products were evaluated in two ways: a) by immersing the sclerotia in the properly prepared mixture for a period of five seconds, b) by spraying the mixture onto the sclerotia positioned on the surface of the soil inside acrylic boxes. In the immersion method, the sclerotia were subsequently seeded in water agar medium, ten per Petri dish. In the spraying method, twenty-five sclerotia were distributed inside each acrylic box. In both methods, 200 sclerotia were analyzed per treatment. The sclerotia were incubated for a period of 45 days at a temperature of 16ºC for twelve hours without light and 20ºC for twelve hours with light. After this period, germinated sclerotia were counted. The results indicated that, when the treatments were applied directly to the sclerotia (immersion in syrup), carpogenic germination was reduced, with the treatments Dimoxystrobin + Boscalid and Carbendazim standing out among the best results, while the treatments Procymidone and *Trichoderma harzianum* 1306 obtained control efficiency similar to the control, while the others presented intermediate performance. However, this effect was not repeated when the products were applied to the sclerotia positioned on the ground. It was also observed under the conditions of this experiment that treatments containing biocontrol agents were not efficient in reducing the carpogenic germination of the fungus and none of the methodologies evaluated.
Keywords: Sclerotium. Viability. Chemical control. Biological control.

INTRODUCTION

Soy is the most cultivated oilseed in the world, with an area of more than 122.6 million hectares and a production of 337 million tons, with Brazil today being the largest producer in the world, with more than 36 million hectares and a production 124.950 million tons (USDA, 2020). In this scenario, Santa Catarina ranks as the 12th largest producing state, with a cultivated area of 687.6 thousand hectares and production of 2,253 thousand tons of grains (EPAGRI/CEPA 2020).

Diseases play a decisive role as productivity-reducing agents in soybean cultivation, among which white mold caused by the fungus stands out.: *Sclerotinia sclerotiorum* (Lib.) by Bary. This fungus is widely distributed throughout producing regions around the world, both in temperate and tropical climates (CAB, 2005). Some of the characteristics that make this fungus so aggressive are its wide host range (PURDY, 1979), ability to produce survival structures (sclerotia) (STEADMAN, 1983), its transmissibility via seeds (DHINGRA, 2005) and the lack of resistant cultivars (JULIATTI et al., 2014).

The disease known as white mold frequently occurs in regions with altitudes above 600 meters, in rainy years and with average temperatures ranging between 15 and 25 degrees during the flowering and pod formation phase (ABAWI & GROGAN 1975). When sclerotia is present in the area and the soil has moisture 50% higher than field capacity for a period of 10 to 20 days and temperatures between 10 and 21ºC, apothecia

are formed (BOLTON et al., 2006).

The apothecia release ascospores that are disseminated by the wind and need to find an exogenous source of nutrients and a film of water to start the infection process (BOLAND & HALL, 1987).

Clarkson et al. (2003) consider that sclerotia represents the main source of inoculum and is also a fundamental part of the pathogen's life cycle; however, the basis of disease control today consists of protecting the pathogen's infection sites through chemical products (SUMIDA et al., 2015; WUTZKI et al., 2016). Although fungicides provide partial protection to crops, there is no information that they provide control of resistance structures present in the soil (TU, 2003). As it is a monocyclic disease, the reduction of the initial inoculum (sclerotia) in the soil is a crucial factor for its control (NAPOLEÃO et al., 2005), since spread from plant to plant may occur occasionally, but is generally a rare occurrence (LINK & JOHNSON, 2007).

An alternative to reducing the source of inoculum in the soil is the use of biological control agents (CARDOSO et al., 2017), such as fungi of the genus: *Trichoderma* spp. (GÖRGEN et al., 2009; ZANCAN et al., 2012) e bactérias do gênero *Bacillus* spp. (GABARDO, et al., 2020; DORIGHELLO, 2017). In some cases, the leaflet of the chemical pesticide itself suggests that it exerts control over the germination of sclerotia (SPOT [Bula], Guaratinguetá/SP, 2017). Assuming that chemical fungicides can exert some suppression of the carpogenic germination of sclerotia and/or the formation of apothecia, the question remains: how much of this applied product will be able to reach the soil and come into contact with the sclerotia?

Taking into consideration, everything previously mentioned, this work was carried out under controlled environmental conditions with the aim of: a) establishing whether there is control of sclerotia through the reduction of carpogenic germination when using chemical fungicides and biocontrol agents; b) check whether this possible control is maintained when the sclerotia are positioned on the ground.

MATERIAL AND METHODS

The sclerotia of *S. sclerotiorum* were obtained from a Seed Processing Unit (UBS) in the municipality of Bom Jesus, state of Rio Grande do Sul, collected from the mass of residue from the processing of soybean seeds cultivar BRS 5601RR harvested in April 2019.

Approximately ten kilos of this residue were taken to the Phytopathology Laboratory of the Agroveterinary Science Center (LF-CAV/UDESC) where 500 g of sclerotia were manually separated. A second separation was carried out to choose and separate sclerotia measuring between 4 and 6 mm in length until weighing 300 g of sclerotia. These sclerotia were disinfected in a 1% sodium hypochlorite solution for five minutes, followed by rinsing in sterile distilled water, and distributed over three layers of germination paper that were placed inside the laminar flow chamber to dry the sclerotia. Every hour, for six hours, the germination paper was replaced in order to reduce humidity and accelerate the drying of the sclerotia, which remained for 24 hours inside the laminar flow chamber. After drying, 24 samples each containing 20 g of sclerotia were separated, with 12 samples to be used in the sclerotia germination test in water agar medium and 12 samples in sterile soil.

The treatments used were chemical fungicides (seven) and biological fungicides (three) indicated for the control of white mold (Table 4), and two control treatments, one with water application and the other without water application.

The effect of the products on the germination of sclerotia was quantified using

Fungicide		Dose of Commercial Product1
Active ingredient	Commercial Product	
Methyl thiophanate (37,5%) + Fluazinam (37,5%)	Approve	1,0 Kg
Fluazinam (50%)	Frowncide	1,0 L
Dimoxystrobin (20%) + Boscalid (20%)	Spot	1,0 L
Methyl thiophanate (87,5%)	Cercobin	0,7 Kg
Bixafen (12,5%) + Protioconazole(17,5%)+Trifloxixtrobina (15,0%)	Fox Xpro	0,5 L
Procimidona (50%)	Sumilex	1,0 Kg
Carbendazim (50%)	Carbendazim Nortox	1,0 L
Trichoderma harzianum 1306 (4,8%)	Trichodermil	1,0 L
Trichoderma harzianum cepa T-22 (1%)	Trianum DS	1,5 L
Bacillus subtilus linh. QST 713	Serenade	2,0 L

Table 1. Fungicides applied to Sclerotinia sclerotiorum sclerotia. Lages, SC, 2020.

[1]Dose indicated by the company that owns the product based on an application with a spray volume of 150 L per hectare.

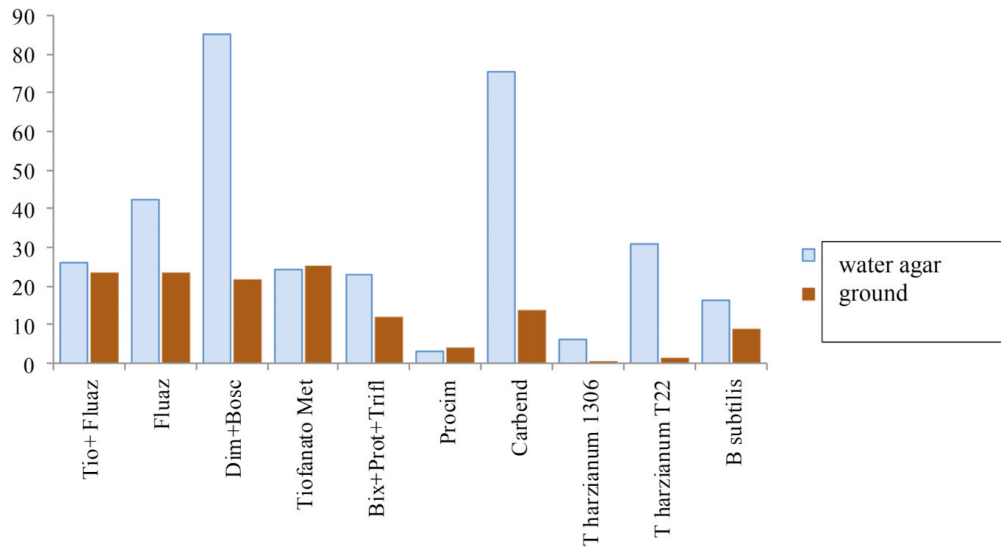

Figure 2. Comparative control efficiency of the treatments used in the sclerotia germination experiments of *Sclerotinia sclerotiorum* in water agar and soil culture media.

two methods: i) immersion of the sclerotia in the fungicide mixture with subsequent distribution on an agar-water medium; ii) spraying fungicides on sclerotia maintained on the sterile soil surface.

The fungicides were dosed according to the instructions (Table 4) in 500 mL of distilled and sterile water contained within a 1-liter Erlenmeyer flask with manual stirring performed with a glass rod. The fungicides were dosed using a precision pipette and/or weighed on an analytical balance and mixed with water. Twenty grams of sclerotia placed inside a 10 x 10 cm nylon bag with a 0.25 mm mesh (Figure 5 A) were immersed for five seconds in the fungicide syrup, being immediately removed with manual shaking to drain the excess syrup and placed inside Petri dishes and shaken gently to be distributed. The plates were taken into the laminar flow chamber where the sclerotia were distributed in other Petri dishes containing water agar medium (1%) (Figure 5 B). With the aid of sterile tweezers, flamed in a lamp, 100 sclerotia

were chosen to be sown on the culture medium, ten sclerotia per plate. The plates were identified, sealed with Parafilm and taken to a BOD (Biological Oxygen Demand) growth chamber, where they remained incubated for a period of 45 days at a temperature of 16ºC for twelve hours without light and 20ºC for twelve hours with white fluorescent light.

The other method of applying fungicides was by spraying the mixture onto the sclerotia positioned on the soil surface inside the acrylic boxes (Figure 5 C). The soil was composed of agricultural substrate (Maxfértil) which was sterilized twice in an autoclave at 121ºC for 20 minutes with a time interval of 48 hours between each sterilization process.

The sterile substrate was distributed in a layer approximately two centimeters high inside gerbox-type acrylic boxes measuring 11 x 11 x 3.5 cm. In each box, 25 sclerotia were distributed, five lines with five sclerotia, four acrylic boxes arranged in a square were sprayed, totaling 100 sclerotia sprayed per treatment. A pressurized sprayer was used (CO_2) with flow to 150 L ha^{-1} applying at a distance of 50 cm above the plates. Subsequently, the boxes were closed and transported to the growth chamber (Figure 5 D).

A)

B)

C)

D)

Figure 1. Detail of the sclerotia inside the nylon bags before being submerged in the treatments (A); plating of sclerotia in water agar culture medium in a laminar flow chamber (B); sclerotia positioned on the surface of the substrate before being pulverized by treatments (C); and gerbox containing the treatments inside the growth chamber (D).

Source: Prepared by the author (2020).

The two assays were carried out on the same day, with the sclerotia incubated in the same growth chamber maintaining the same temperature variables, light regime and incubation time period. The Petri dishes and acrylic boxes were randomly distributed on the inner shelves of the chamber, being randomly redistributed every five days. After the incubation time, the plates and boxes

were removed from the growth chamber and positioned next to a stereoscopic magnifying glass. The sclerotia from all treatments were visualized to observe the presence of stipes and apothecia that characterized germination. Sclerotia were considered germinated if they had a stipe greater than or equal to one mm (Figure 2A) and an apothecium opening with a diameter greater than or equal to one mm (Figure 2B).

The data obtained were submitted to ANOVA 5% and subsequently to the means comparison test by Tukey 5% using the Sasm-Agri application (CANTERI, et al., 2001). The results were expressed as percentage of germination, considering the presence of stipe (with and without apothecium) or presence of apothecium. The control efficiency of each treatment was also calculated according to the equation: E.C% = , the two controls were used separately for the calculation (with and without the addition of water) and then the average of both was calculated.

RESULTS AND DISCUSSION

In the first experiment, the ability of agricultural pesticides to suppress the carpogenic germination of the fungus was evaluated, subjecting the sclerotia to an extreme situation, direct immersion in the syrup with the products. For comparison purposes, two controls were carried out, one submerged in water and the other not, however the results of the two did not differ significantly, with the percentage of carpogenic germination of sclerotia being 59% and 63% respectively (Table 5).

According to the results obtained, treatment (3) Dimoxystrobin + Boscalid was the one that showed the best efficacy, with only 9% germination, followed by (7) Carbendazim with 15%. A group with five products was observed, namely: (1) Methyl thiophanate + Fluazinam, (4) Methyl thiophanate, (5) Bixafen + Prothioconazole + Trifloxytrobin, (9) *Trichoderma harzianum* cepa T-22 (1%), (10) *Bacillus subtilus* linh. QST 713 which showed similar efficiency to both the controls and the treatment (2) Fluazinam. The treatments (6) Procymidone and (8) *Trichoderma harzianum* 1306 (4,8%) did not differ from the witnesses (Table 5).

	Treatment	G.E		E.C (%)	E.C(%)	E.C.M[3]
		%		test. c/ H_2O	test. s/ H_2O	%
1	Tiof+Fluaz	45	ab	24	29	26
2	Fluazinam	35	bc	41	44	43
3	Dim+Bosc	9	d	85	86	85
4	Tiofanto Met	46	ab	22	27	25
5	Bix+Prot+Trifl	47	ab	20	25	23
6	Procimidona	59	a	0	6	3
7	Carbendazin	15	cd	75	76	75
8	Trichodermil	57	a	3	10	6
9	Trianum	42	ab	29	33	31
10	Serenade	51	ab	14	19	16
11	Test c/H_2O[1]	59	a	0	0	0
12	Test. s/H_2O[2]	63	a	0	0	0
	C.V%	33,12				

Table 1. Percentage of sclerotia germination (G.E), control efficiency (E.C) of fungicides compared to the control in the presence and absence of water and the average control efficiency (E.C.M) considering sclerotia incubated in agar-water culture medium. Lages, 2020.

Means followed by the same letter in the line do not differ from each other using the Tukey test p=0,05.

[1]Sclerotia immersed in water before being sown on water agar; 2Sclerotia not immersed in water and sown in water agar; 3medium control considering sclerotia immersed or not in water.

Contrasting results were described by Oliveira et al. (1999), where the authors observed that Fluazinam and Vinclozin

were more than 90% efficient in reducing carpogenic germination of sclerotia when they were submerged for one minute in the solution containing the respective fungicides. Carbendazim had an efficiency of only 25%, not differing from the control, procymidone showed an intermediate efficiency of approximately 45%. However, there is a difference regarding the time of exposure of sclerotia to the fungicide between the two studies, being one minute in the case of Oliveira et al. (1999) and five seconds in this one.

Similar to this study, Sumida et al. (2014) observed that Carbendazin with a dosage above 1.0 µL of formulated product for each liter of culture medium (BDA), reduced the mycelial growth of *Sclerotinia sclerotiorum* in vitro. However, methyl thiophanate at the same concentration only showed a 42% reduction. Just as we observed that most of the treatments evaluated showed some ability to reduce the carpogenic germination of structures, Zancan et al. (2012) evaluating the in vitro sensitivity of two isolates of *Sclerotinia sclerotiorum* subjected to several chemical fungicides and a biocontrol agent, observed that all treatments used were able to reduce the mycelial growth of the fungus, however, when the sclerotia were produced in a culture medium containing the evaluated products, they all had the capacity to germinate carpogenically independent of the treatment and concentration used.

Table 6 describes the results relating to the second experiment, where the sclerotia were positioned in moist soil inside gerbox-type boxes and then the treatments were sprayed at their respective commercial doses, simulating the effect of pesticides applied in the field.

	Treatment	G.E %		E.C (%) test. c/ H_2O	E.C(%) test. s/ H_2O	E.C.M[3] %
1	Tiof+Fluaz	47	A	22	25	24
2	Fluazinam	47	A	22	25	24
3	Dim+Bosc	48	A	20	24	22
4	Tiofanto Met	46	A	23	27	25
5	Bix+Prot+Trifl	54	A	10	14	12
6	Procimidona	59	A	2	6	4
7	Carbendazin	53	A	12	16	14
8	Trichodermil	64	A	-7	-2	-4
9	Trianum	61	A	-2	3	1
10	Serenade	56	A	7	11	9
11	Test c/H_2O[1]	60	A	0	0	0
12	Test. s/H_2O[2]	63	A	0	0	0
	C.V%	22,17				

Table 2. Percentage of sclerotia germination (G.E), control efficiency (E.C) of fungicides compared to the control in the presence and absence of water and the average control efficiency (E.C.M) considering sclerotia incubated in soil culture medium. Lages, 2020.

Means followed by the same letter in the line do not differ from each other using the Tukey test p=0,05.

[1]Sclerotia sprayed only with distilled water after being placed in the soil; [2]Sclerotia not sprayed with distilled water after being placed in soil;

[3] medium control considering sclerotia sprayed or not with water.

A sharp drop in the efficiency of the evaluated products was observed in reducing the germination of the fungus' resistance structures when they were applied via soil, none of which differed from the control. The data obtained here corroborate those observed by Vriesman et al. (2014) in an experiment carried out in a similar way to this one, where the active ingredients fluazinam, carbendazin, and methyl thiophanate obtained sclerotia germination rates similar to the control. The authors also attest that the

fungicide Dimoxystrobin + Boscalid was the most efficient treatment, reducing the rate of carpogenic germination of sclerotia in the experiment from 96 to 68%, that is, a gain of 28%, similar to this work, where the gain was approximately 39%.

In another similar study, where fungicides were applied to sclerotia buried two centimeters in the soil under controlled conditions, Costa and Costa 2007 observed that the fungicides thiophanate-methyl and procymidone were 75% efficient in reducing carpogenic germination, while the fungicide fluazinam did not. was efficient in reducing carpogenic germination, but obtained 100% in inhibiting the formation of the apothecium.

A detail that differs between the two methodologies is that in this case, in addition to being buried two centimeters in the soil, the fungicides were applied to the soil, simulating a water depth of 6.0 millimeters per hectare.

A situation that drew attention was in relation to the poor performance of the antagonistic microorganisms used in the experiment, whose representatives are fungi of the genus: *Trichoderma* and bacteria of the genus: *Bacillus*, not differing from the witnesses in any of the methodologies evaluated. In a study evaluating five isolates of *S. sclerotiorum, Moraes and Carvalho, (2015) also did not observe antagonism in vitro* of *Trichoderma* sppfor none of them. In a similar way, but at the field level, Meyer et al., (2019b) observed that no commercial biofungicide evaluated inhibited the carpogenic germination of sclerotia in six evaluated locations, with the microorganisms being evaluated; *T. asperellum, T. harzianum and B. subtillis*.

The authors point out as the main reasons for the low efficiency of biocontrol agents in the field, the irregularity of rainfall at the time of application and the low availability of straw in the evaluated locations. Along the same line of reasoning, Görgen et al., (2009) observed greater parasitism by *Trichoderma harzinanum* in sclerotia when in the presence of straw in the soil.

After the sclerotia were exposed to their respective treatments, they were immediately taken to BOD where they remained for 45 days with a 12-hour photo period. This could be a reason to explain the low effectiveness of biocontrol agents, however Milan et al. (2015) found that effectiveness in biological control of *Trichoderma* is more linked to the nature of the isolate than to the light regime to which it is subjected.

There are many ways in which the *Trichoderma* works to control the *Sclerotinia sclerotiorum*, among which mycoparasitism and antibiosis stand out, which is the production of metabolites and enzymes that degrade the cell wall, which have great potential for control (MONTE et al., 2019). However, unlike what happens with a chemical product, the biocontrol agent needs more time to express its antagonistic potential, since it is applied to the sclerotia in the form of colony forming units (CFU) or conidia. In this aspect, Troian (2014) reports that only after six days of incubation, the presence of sporulation of *Trichoderma* about isolates of *Sclerotinia sclerotiorum.*

Similar results were described by Monteiro et al. (2013) who observed a greater presence of bacterial cells of *Bacillus subtilis* in sclerotia previously incubated for 6 days, which also provided, according to the authors, inhibition of mycelial and carpogenic germination.

In a similar way, Venturini et al. (2014) observed better efficiency of *Bacillus subtilis* in reducing mycelial growth of *Thielaviopsis paradoxa* in relation to the control, when the previous incubation took place within 48 hours. The efficiency of the microorganism was reduced when the incubation time was 24 and 0 hours respectively. In an experiment

aiming to evaluate the effect of antagonistic agents on the growth of phytopathogens in vitro, Gabardo et al. (2020) found that *Bacillus subtilis* was efficient in controlling *S. sclerotiorum*, however, it did not prevent the formation of sclerotia by the same.

Another pertinent information raised in these two experiments can be seen in figure 6 and concerns the reduction in the control efficiency of treatments applied via soil, when compared to direct immersion of the sclerotia in the syrup containing the respective products.

It can be noted that with the exception of the Thiophanate methyl and procymidone treatments, all others had reductions in the rate of control of sclerotia germination when applied via soil. The most striking examples were in the case of the treatment Dimoxystrobin + Boscalid and Carbendazin, which had more than 60% reduction in efficiency. This shows that, although many products used to control S. sclerotiorum in aerial parts have an effect on the germination of sclerotia, we cannot always use this to our advantage in the field, given the difficulty of making the products reach the target below ground. Holtz et al. (2014), demonstrate that only 16% of the volume of syrup applied to a soybean crop, with 0.5 meter spacing between rows, managed to reach the leaves of the bottom. It can be assumed that the volume of spray that reaches the ground is even smaller.

CONCLUSIONS

The products Dimoxystrobin + Boscalid and Carbendazin were efficient in reducing the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* when these were immersed in the respective syrups.

No product evaluated in this experiment was effective in reducing the carpogenic germination of the fungus when applied to sclerotia positioned on a substrate.

The biological fungicides evaluated in this trial were not efficient in reducing the carpogenic germination of fungal structures.

On average, the products sprayed on the sclerotia in the substrate lost 20% in efficiency compared to the syrup immersion methodology.

FINAL CONSIDERATIONS

The experiments conducted during the execution of this work show us that, although a certain active ingredient may be efficient in controlling sclerotia germination in the laboratory, it does not mean that this will be repeated in the field, as there are many factors involved, such as the position of the sclerotia. in the soil and the amount of product that can reach it.

A suggestion for future laboratory experiments with biocontrol agents on sclerotia would be to evaluate incubation periods, for example, zero, five, ten days before placing the sclerotia to incubate and stimulate carpogenic germination. It is known that the response time of a biological control agent is in most cases greater than that of a chemical product, so much so that at the field level, it is recommended to apply *Trichoderma* or *Bacillus* at least 15 days before the soybeans flower, which is the time when the plant is most prone to attack by the pathogen.

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