In vitro sensitivity of *Sclerotinia sclerotiorum* isolated from soybeans to fungicides

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ABSTRACT

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In *in vitro* experiments conducted in the laboratory, the sensitivity of *Sclerotinia sclerotiorum* isolated from soybean, to boscalid, carbendazim, fluazinam, procymidone, tebuconazole and thiophanate methyl was determined. To found the 50% inhibitory concentration (IC_{50}) of mycelial growth, the potato-sucrose agar medium was supplemented with 0.0, 0.01, 0.10, 1.0, 25.0 and 50.0 mg/L of active ingredient, poured into petri dishes and observing four replications. Discs containing mycelium and substrate, 6.0 mm in diameter, were deposited in the dishes center and kept for seven days in a growth chamber at 18 ± 2°C and 12 h photoperiod. The chemical effect on the mycelial growth was determined by measuring the colonies diameter. The data, as percent inhibition relative to the concentration zero, were submitted to logarithmical regression and with the generated equation, the IC_{50} was calculated. IC_{50} ranged from <0.01 for the fluazinam, 0.09 for carbendazim, 0.13 for tebuconazole, 0.26 for procymidone, 0.29 for boscalida and 42 mg/L for thiophanate methyl. It was confirmed that fluazinam, a multisite, protectant was the most potent fungicide against *S. sclerotiorum*.

Keywords – Fluazinam, *Glycine max*, white mold, mycelial sensitivity.

RESUMO

Brustolin, R.; Reis, E. M. Sensibilidade *in vitro* de *Sclerotinia sclerotiorum*, isolados de soja, a fungicidas. **Summa Phytopathologica,** v. n. p. 00-00, 2015.

Em experimentos conduzidos *in vitro* em laboratório, determinou-se a sensibilidade de *Sclerotinia sclerotiorum,* isolados de soja, aos fungicidas boscalida, carbendazim, fluazinam, procimidona, tebuconazol e tiofanato metílico. Para a determinação da concentração inibitória de 50% (CI_{50}) do crescimento micelial, o meio de cultura de batata-sacarose-ágar foi suplementado com 0,0; 0,01; 0,10; 1,0; 25,0 e 50,0 mg/L de ingrediente ativo, vertido em placas de Petry e observando-se quatro repetições. Discos contendo micélio e substrato, de 6,0 mm de diâmetro, foram depositados no centro dos recipientes e mantidos por sete dias em câmara de crescimento a 18 ± 2^{o}C e fotoperíodo de 12h. O efeito dos tratamentos sobre o crescimento micelial foi determinado pela mensuração do diâmetro das colônias. Os dados da percentagem de inibição em relação à concentração zero, foram submetidos a regressão logarítmica e com a equação gerada calculada a CI_{50}. A CI_{50} variou de < 0,01 para o fluazinam, 0,09 para o Carbendazim, 0,13 para o tebuconazol, 0,26 para a procimidona, 0,29 para a boscalida e 0,42 mg/L para o tiofanato metílico. Confirmou-se que o fungicida multissítio, protetor mais potente inibidor do micélio de *S. sclerotiorum* foi o fluazinam.

Palavras-chave – Fluazinam, *Glycine max*, mofo branco, sensibilidade micelial.

INTRODUCTION

The Brazilian soybean crop [*Glycine max* (L.) Merr] cultivated in 2013-14 covered an area of 29.8 million hectares and yielded 2.8 t/ha, resulting in an overall production of 90 million tons (3).

Sclerotinia stem rot (SSR), or white mold (WM) of soybean, is a significant yield-limiting disease in Brazil. This disease, caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is among the most nonspecific, omnivorous, and successful of plant pathogens. Plants susceptible to this pathogen reach 652 species (nt.ars-grin.gov/fungaldatabases/index.cfm).

WM varies in incidence and severity from season to season according to weather conditions. Considering white mold area with the highest incidence, the estimated infested acreage in 2008 was 4%, rising to 6.5% in 2009, 9.8% in 2010, 14.4% in 2011, 22.9% in 2012 and reaching 25.5% in 2013 (11).

Soybean cultivars with resistance/tolerance to Ss are not yet available and the soybean area is grown under monoculture which contributes to increase the sclerotia bank in the soil, season after season. Crop rotation with nonsuseceptible crops could reduce the inoculum in the soil of cultivated area but it is not follow by growers (2).

Losses caused by SSR can be appraised by the damage function y = - 6.7 x + 1,000, where y represents grain yield normalized to 1,000 kg/ha and x represents SSR incidence in plants (13).

An effective disease management strategy integrates several tactics that include cultural practices, as chemical and biological control. However, chemical is the most used.

Boscalid, carbendazim, iprodione, fluazinam, fludioxonil, procymidone, tiophanate methyl, and vinclozolin, are among the most used fungicides to control SSR especially in canola, edible beans, peanuts and soybean (7, 8, 9, 10, 12, 18).

The objective of this work was to identify the most potent fungicides among those reported with the highest fungitoxicity towards *S. sclerotiorum* isolates to be used in an antiresistance strategy.

MATERIAL AND MEGTHODS

Sclerotinia isolates. To evaluate the mycelial growth of Ss to fungicides, the chemicals were incorporated in an agar medium, similar to the method described by Russel (5). Five selected monoascosporic isolates (CX, from Coxilha county Rio Grande do Sul state, SC, from Santa Catarina state, BA, from Luiz Eduardo Magalhães county Bahia state, PR, from Faxinal county Paraná state, and GO, from Rio Verde country, Goias state) were preserved in test tubes with PSA (40 g potatoes, 10 g sucrose, 14 g agar in 1,000 mL distilled water) medium in a refrigerator at 5°C and used throughout this work.

Fungicides. Six chemicals, containing carbendazim (Bendazol 500 SC), fluazinam (Frowncide 500 SC), boscalid (Cantus 500 WG), procymidone (Sumilex 500 WP), and tebuconazole (Folicur 200 EC), were assessed for Ss mycelial growth inhibition.

Substrate and fungicide concentrations. Five days old colonies of each strain were grown on PSA, supplemented with the fungicides after sterilization in an autoclave. Seven concentrations of each fungicide were used as follows: 0.0, 0.01, 0.10, 1.0, 10.0, 25.0 and 50.0 mg/L, with four replications. The day after the media preparation, mycelial discs (6 mm diameter) of each isolate were placed upside down on the center of each Petri dish. The plates were sealed with PVC film and incubated in a growth chamber at 18 ± 2ºC and 12 h photoperiod for seven days.

Assessment. Colony growth (mm) in two perpendicular diameters were measured with a digital caliper (Stainless Worker), when the fungal growth in the control treatment had reached the plate edge. Means of the two diameters measurements were used and converted to percent growth and compared with the fungal growth in the control treatment (0.00 mg/L).

Statistical procedure. Logarithmical regression analysis using the statistical program Costat were performed. The inhibitory concentration (IC_{50}) capable of inhibiting 50% of Ss mycelial growth for each isolate and fungicide was calculated from the generated equation. The experimental design was a completely randomized, with four replications, each experimental unit consisting of a Petri dish.

Experiments were repeated twice to ensure accuracy.

The isolate sensitivity was classified according to the standard criteria of Edgington et al. (4), with the following attributes: insensitive when IC_{50} >50 mg/L; moderately sensitive when IC_{50} between 1 and 10 mg/L; highly sensitive when IC_{50} <1 mg/L. The IC_{50} describe the active ingredient concentration, which inhibit 50% of the mycelial growth or of the spore germination.

RESULTS AND DISCUSSION

For fluazinam, a piridinamine, multisite, nonpenetrant, protectant fungicide, IC_{50} was < 0.01 mg/L for the five isolates and r^2 ranged from 0.73 to 0.84 (Table 1). Similarly, Matheron et al. (2004) found that at 0.01 mg/L *S. minor* was reduced from 82 to 84% by fludioxonil and fluazinam. Sumida et al. (19) and Zancan et al. (23) also reported the best performance of fluazinam among the tested fungicides. Fluazinam has also been used with success in the field to control WM in beans and peanuts (18).

Table 1. Isolate, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by fluazinam

Isolate	Equation*	r^2	IC_{50}**	*p*
CX	y=2.27ln(x)+93.5	0.84	<0.01	<0.0001
SC	y=2.79ln(x)+92.45	0.75	<0.01	<0.0001
BA	y=3.44ln(x)+90.96	0.73	<0.01	<0.0001
PR	y=2.53ln(x)+92.58	0.80	<0.01	<0.0001
GO	y=3.40ln(x)+91.14	0.73	<0.01	<0.0001
Media	y=2.89ln(x)+92.13	0.76	<0.01	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation.

For carbendazim, a benzimidozole, site specific, penetrant, mobile fungicide, IC_{50} ranged from 0.05, PR isolate to 0.14 mg/L, BA isolate and r^2 ranged from 0.67 to 0.80 with a mean of 0.09 mg/L (Table 2). Wang et al (24) and Yong et al. (22) have reported the Ss resistance to carbendazim a high risk fungicide belonging to the benzimidazole group such as tiophanate methyl and benomyl (6).

Although showing a high fungitoxicity towards Ss, under field application should be take into consideration the sensitivity loss by *Corynespora cassiicola* (Berk. & M.A. Curtis) Wei, isolates from soybean, to carbendazim (1).

Table 2. Isolates, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by carbendazim

Isolate	Equation*	r^2	CI_{50}**	*p*
CX	y=10.01ln(x)+70.87	0.76	0.12	<0.0001
SC	y=9.02ln(x)+72.61	0.67	0.08	<0.0001
BA	y=9.78ln(x)+69.48	0.75	0.14	<0.0001
PR	y=7.81ln(x9+73.38	0.80	0.05	<0.0001
GO	y=9.22ln(x)+71.66	0.67	0.10	<0.0001
Media	y=9.17ln(x)+71.6	0.73	0.09	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation.

For the DMI site specific, penetrant, mobile, tebuconazole, IC_{50} ranged from <0.01, for CX isolate to 0.87 mg/L, SC isolate and with a mean of 0.13. In addition, r^2 ranged from 0.67 to 0.95 (Table 3). This chemical should not use alone in soybean due to its sensitivity reduction towards *Phakopsora pachyrhizi* Sydow (14, 17).

Table 3. Isolates, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by tebuconazole

Isolado	Equação*	r^2	CI_{50}**	*p*
CX	y=5.09ln(x)+87.25	0.67	<0.01	<0.0001
SC	y=13.10ln(x)+51.81	0.92	0.87	<0.0001
BA	y=11.58ln(x)+63.73	0.95	0.31	<0.0001
PR	y=8.50ln(x)+76.21	0.82	0.05	<0.0001
GO	y=9.77ln(x)+68.21	0.91	0.16	<0.0001
Media	y=9.61ln(x)+69.44	0.94	0.13	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation.

Regarding procymidone, a dicarboxamide, nonpenetrant, protectant fungicide, IC_{50} ranged from 0.15, SC isolate to 0.40 mg/L for BA isolate, with a mean of 0.26 mg/L, and r^2 ranged from 0.81 to 0.88 (Table 4). The activity of dicarboximide fungicides was first reported in the early 1970's with the three key commercial products being introduced within three years; iprodione, vinclozolin and procymidone.

Table 4. Isolates, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by procymidone

Isolado	Equação*	r^2	CI_{50}**	*p*
CX	y=10.15ln(x)+71.38	0.81	0.20	<0.0001
SC	y=10.35ln(x)+69.87	0.85	0.15	<0.0001
BA	y=13.15ln(x)+62.03	0.85	0.40	<0.0001
PR	y=12.46ln(x)+62.61	0.88	0.36	<0.0001
GO	y=12.83ln(x)+63.06	0.85	0.36	<0.0001
Media	y=11.79ln(x)+65.70	0.87	0.26	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation

Considering boscalid, a site specific carboxamide fungicide, nonpenetrant, protectant fungicide, IC_{50} ranged from 0.11, PR isolate to 0.57 mg/L, GO isolate, with a mean of 0.29, and r^2 ranged from 0.73 to 0.84 (Table 5). The IC_{50} found by Xin Liu et al. (21) for boscalid ranged from 0.002 to 0.391 mg/L with a mean of 0.042 mg/L, 6.0-fold lower than we found.

Table 5. Isolates, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by boscalid

Isolate	Equation*	r^2	IC_{50}**	*p*
CX	y=10.31ln(x)+60.38	0.94	0.37	<0.0001
SC	y=9.26ln(x)+60.66	0.88	0.32	<00001
BA	y=8.86ln(x)+63.07	0.93	0.23	<0.0001
PR	y=8.20ln(x)+68.45	0.89	0.11	<0.0001
GO	y=9.04ln(x)+55.04	0.93	0.57	<0.0001
Mean	y=9.22ln(x)+61.52	0.96	0.29	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation.

In relation to tiophanate methyl, also a benzimidazole, IC_{50} ranged from 0.19, BA isolate to 0.54 mg/L, GO isolate, with a mean of 0.42, and r^2 ranged from 0.83 to 0.90 (Table 6). The first case of Ss resistant to benomyl, also a benzimidazol, was reported by Gossen et. al. (6).

Table 6. Isolates, equation, coefficient of determination (r^2), fungicide concentration to inhibit 50% (IC_{50}) the mycelial growth of *Sclerotinia sclerotiorum* by tiophanate methyl

Isolado	Equação*	r^2	IC_{50}**	*p*
CX	y=12.40ln(x)+59.63	0.90	0.46	<0.0001
SC	y=12.21ln(x)+58.07	0.86	0.52	<0.0001
BA	y=10.31ln(x)+67.32	0.85	0.19	0.0011
PR	y=10.97ln(x)+57.64	0.86	0.50	<0.0001
GO	y=12.74ln(x)+57.82	0.83	0.54	<0.0001
Media	y=11.73ln(x)+60.1	0.86	0.42	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation

Xin et al. (21) determined for iprodione IC_{50} ranged from 0.163 to 0.734 μg/ml with a mean of 0.428 μg/ml similar to our findings for tiophanate methyl.

The overall mean for fungitoxicity ranged from <0.01 for fluazinam, the most fungitoxic, to 0.42 mg/L for tiophanate methyl the least potent, and r^2 ranged from 0.73 to 0.96 (Table 7). Figueiredo reported tiophanate methyl as highly toxic but at 1 mg/L, a concentration 2.38-fold higher than the concentration here tested.

Table 7. Fungicide, equation and fungicide concentration to inhibit 50% (IC_{50}) mycelial growth of *Sclerotinia sclerotiorum*, means of five isolates

Fungicide	Equation*	r^2	**CI_{50}****	p**
Fluazinan	y=2.89ln(x)+92.13	0.76	**< 0.01**	<0.0001
Carbendazim	y=9.17ln(x)+71.6	0.73	**0.09**	<0.0001
Tebuconazole	y=9.61ln(x)+69.44	0.94	**0.13**	<0.0001
Procymidone	y=11,79ln(x)+65.70	0.87	**0.26**	<0,0001
Boscalid	y=9.22ln(x)+61.52	0.96	**0.29**	<0.0001
Tiophanate methyl	y=11.73ln(x)+60.1	0.86	**0.42**	<0.0001

*y = percent mycelial growth inhibition. **Concentration (mg/L) calculated by the equation.

According to Edgington et al (4) all isolates were highly sensitive to the tested fungicides with $CI_{50} < 1.0$ mg/L.

Although not tested in our work, Mueller et al. (12) reported vinclozolin as the fungicide showing the highest fungitoxicity towards Ss. Kuang et al. (9), found the mean IC_{50} values for fludioxonil based on inhibition of mycelial growth of 120 wild-type isolates, 0.015 ± 0.005 µg/ml. Positive cross-resistance was not detected between fludioxonil and benzimidazole fungicides but was detected between fludioxonil and dicarboximide fungicides which are considered as high resistance risk fungicides by FRAC (5).

The search for powerful fungicide to Ss control should be a continuous task. Various fungicides have shown high fungitoxicity to Ss, however, under field conditions, exhibit reduced efficiency. This difference can be attributed to difficulty to protect the Ss infection sites, the senesced petals in the lower third of the plant and protected by innumerous layers of leaves.

Soybean cultivars with resistance/tolerance to Ss are not yet available. The WM infested area continuous to grow, reaching 25.5% in 2013 growing season (11). This large soybean area, grown under monoculture, which contributes to increase the sclerotia bank in the soil, season after season. Crop rotation with nonsuseptible crops could help to reduce the inoculum in the cultivated soil but it is not follow by growers (2).

So far, only fluazinam, procymidone and fluazinam + thiophanate methyl, and thiophanate methyl alone have been registered in the Ministry of Agriculture (http://www.agricultura.gov.br/), for WM control in soybeans.

If the WM control continuous to be based only on the use of fungicides, as is being done, there is the risk of development Ss resistant strains which could aggravate the situation further. Reports were found on the Ss resistance towards carbendazim (22), benomyl (6), and boscalid (24).

Fluazinam is a protectant broad-spectrum fungicide, but is neither systemic nor curative, acts by inhibiting the germination of spores and the development of infection structures. It is classed as a diarylamine or arylaminopyridine and its mode of action involves the compound being an extremely potent uncoupler of oxidative phosphorylation in mitochondria and having high reactivity with thiols.

No report on the Ss resistance to fluazinam was found but only to *Botrytis cinerea* Pers. ex Fries (16).

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