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In vitro mycelial sensitivity of *Macrophomina phaseolina* to fungicides[1]

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RESUMO

Sensibilidade micelial *in vitro* de *Macrophomina phaseolina* a fungicidas

A podridão negra das raízes, causada por *Macrophomina phaseolina* (Tass.) Goid., é a doença radicular mais comum em áreas cultivadas com soja. Este trabalho objetivou determinar a sensibilidade micelial *in vitro*, medida pela CI_{50} (concentração para inibir 50% do crescimento miceliano do fungo) de um isolado de *M. phaseolina* obtido de soja, a diferentes fungicidas (thiram, iprodione, carbendazim, piraclostrobina, fluquinconazole, tolifluanida, metalaxil e penflufen + trifloxistrobina), em seis concentrações (0,01 mg L^{-1}; 0,10 mg L^{-1}; 1,00 mg L^{-1}; 10,00 mg L^{-1}; 20,00 mg L^{-1}; e 40,00 mg L^{-1} do ingrediente ativo). A concentração de 0,00 mg L^{-1} representou a testemunha, sem adição de fungicida. A avaliação do crescimento miceliano foi realizada com o auxílio de paquímetro digital, medindo-se o diâmetro das colônias, quando o crescimento do fungo no tratamento testemunha atingiu a borda da placa de Petri. O delineamento experimental foi inteiramente casualizado, com quatro repetições. Quanto à fungitoxicidade dos ingredientes ativos, evidenciou-se variação de atóxicos a altamente fungitóxicos, para o isolado de *M. phaseolina*, com valores para CI_{50} situando-se entre 0,23 mg L^{-1} e > 40,00 mg L^{-1}, sendo o carbendazim o mais eficiente (CI_{50} = 0,23 mg L^{-1}). O fungo apresentou insensibilidade aos ingredientes ativos fluquinconazole, metalaxil, tiram e tolifluanida.

PALAVRAS-CHAVE: *Glycine max* L.; podridão radicular; fungitoxidade; CI_{50}.

ABSTRACT

Black root rot, caused by *Macrophomina phaseolina* (Tass.) Goid., is the most common root disease in soybean fields. This study aimed to determine the *in vitro* mycelial sensitivity, measured by the IC_{50} (concentration to inhibit 50% of the fungus mycelial growth) of a *M. phaseolina* isolate obtained from soybean, to different fungicides (thiram, iprodione, carbendazim, pyraclostrobin, fluquinconazol, tolyfluanid, metalaxyl and penflufen + trifloxystrobin), at six concentrations (0.01 mg L^{-1}, 0.10 mg L^{-1}, 1.00 mg L^{-1}, 10.00 mg L^{-1}, 20.00 mg L^{-1} and 40.00 mg L^{-1} of the active ingredient). The 0.00 mg L^{-1} concentration represented the control, without fungicide addition. The mycelial growth evaluation was performed with the aid of a digital pachymeter, by measuring the colonies diameter, when the fungus growth in the control treatment reached the Petri dish edge. The experimental design was completely randomized, with four replications. Concerning the fungitoxicity of active ingredients, a variation from non-toxic to highly fungitoxic was observed to the *M. phaseolina* isolate, with IC_{50} values ranging from 0.23 mg L^{-1} to > 40.00 mg L^{-1}, being carbendazim the most efficient one (IC_{50} = 0.23 mg L^{-1}). The fungus showed insensitivity to the active ingredients of fluquinconazole, metalaxyl, thiram and tolyfluanid.

KEY-WORDS: *Glycine max* L.; root rot; fungitoxicity; IC_{50}.

INTRODUCTION

Black root rot, popularly known as charcoal rot, is a disease commonly found in soybean fields. This pathogen can infect roots, stems, leaves and pods of different plant species, affecting more than 500 economic crops (Sinclair & Backman 1989, Almeida et al. 2001). Its causal agent is the *Macrophomina phaseolina* (Tass.) Goid. fungus (Sinclair & Backman 1989), which is also known as *M. cajan*, *M. sesame*, *Rhizoctonia bataticola* or *Sclerotium bataticola* (Sartorato & Rava 1994). In Brazil, the pathogen occurrence was first reported in Campinas, São Paulo State, in 1935, infecting bean roots (Coelho Neto 1994).

It is a polyphagous and cosmopolitan fungus that attacks many species of cultivated plants, including soybean, sorghum, peanut, cowpea,

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sesame, sunflower, bean, cotton, black lentil, chili pepper, maize, tall grass prairie, tomato and watermelon, among others, affecting more than 500 plant species (Machado 1980, Singh et al. 1990, Wyllie 1993, Smith & Carvil 1997, Su et al. 2001, Saleh et al. 2010, Mahdizadeh et al. 2011).

This pathogen was detected in the root epidermal tissues, at the maturity stage, in dry weather conditions (Almeida et al. 2003). The ideal temperature for the fungus is 28-32ºC, with temperature, moisture content and number of sclerotia g^{-1} of soil being important factors for its survival (Cardona 2006). According to Singh & Singh (1982) and Santos et al. (1984), for almost all its hosts, the fungus is efficiently transmitted by seeds.

The charcoal rot is a disease whose importance has increased in recent seasons, being favored by high temperatures and water stress. Its control includes the use of clean seeds, as well as their treatment with fungicides. Crop rotation is not considered efficient, since the fungus has competitive saprophytic ability (Almeida et al. 2001, Pearson et al.1984).

Theoretically, the most practical and economical way of controlling the charcoal rot is using resistant cultivars, however, no genotype resistant to this disease has been identified so far (Almeida et al. 2001). In relation to its chemical control, in Brazil, there are no fungicides registered for this pathogen in soybean (Agrofit 2012). Thus, it is necessary to evaluate fungicides and their efficiency for controlling it.

Seed treatment with fungicides is a practice that has been used by an increasing number of farmers who grow soybean. The amount of seeds treated with fungicides, in the 1991/1992 crop season, did not reach 5% of the sown area, and it is currently around 90-95%, in Brazil (Henning et al. 2010). Thus, it is necessary to evaluate the fungicides effectiveness to improve this pathogen control in soybean seeds.

This study aimed at determining the *in vitro* mycelial sensitivity and the IC_{50} values of a soybean *M. phaseolina* isolate to various fungicides, in order to verify the fungicides effectiveness in soybean seed treatments recommended by researchers.

MATERIAL AND METHODS

The experiment was conducted at the Universidade de Passo Fundo (UPF), Rio Grande do Sul State, Brazil, in 2011. The *M. phaseolina* mycelial sensitivity to fungicides was determined in a bioassay with the fungicides incorporation in solidifying potato dextrose agar (Fernandez 1993), similarly to the method described by Avozani (2011). The *M. phaseolina* mycelium growth sensitivity evaluation was performed *in vitro* to eight fungicides (Table 1) tested for a fungal strain isolated from soybean plant roots collected in a farm, in Passo Fundo.

The concentrations of 0.01 mg L^{-1}, 0.10 mg L^{-1}, 1.00 mg L^{-1}, 10.00 mg L^{-1}, 20.00 mg L^{-1} and 40.00 mg L^{-1} of each fungicide active ingredient were used in the bioassay, being the 0.00 mg L^{-1} concentration considered the control, without fungicide addition.

For dilution, aliquots of each fungicide were transferred, with the aid of a micropipette, to a flask containing distilled sterile water (DSW), resulting in a 100 mL final volume (stock suspension 1). From the first fungicide suspension, 1.0 mL was transferred to a 99.0 mL flask containing DSW, considered the second dilution (stock suspension 2). Then, they were added to a dehydrated PDA (potato dextrose agar-merk) culture medium (39 g L^{-1}), after autoclaving

Table 1. Fungicides used to determine the *in vitro* sensitivity of *Macrophomina phaseolina* to a soybean isolate (Passo Fundo, RS, 2011).

Trademark	Active ingredient (a.i.)	a.i. concentration (g L^{-1})	Chemical group
Mayran	Thiram	700 g kg^{-1}	Dimethyldithiocarbamate
Rovral	Iprodione	500 g kg^{-1}	Dicarboximide
Derosal	Carbendazim	500 g L^{-1}	Benzimidazol
Comet	Pyraclostrobin	250 g L^{-1}	Estrobilurin
Atento	Fluquinconazole	167 g L^{-1}	Triazol
Euparen	Tolyfluanid	500 g kg^{-1}	Fenilsulfamide
Ridomil*	Metalaxyl	40 g L^{-1}	Acilalaninate
BYF + TFS**	Penflufen + trifloxystrobin	154 + 154 g L^{-1}	-

Source: Agrofit. * Ridomil Gold Bravo; ** Test product.

and cooling to obtain the desired concentrations, resulting in a 500 mL final volume.

The stock suspension 1 was used to get the 10.00 mg L^{-1}, 20.00 mg L^{-1} and 40.00 mg L^{-1} concentrations, and the stock suspension 2 to obtain the 0.01 mg L^{-1}, 0.10 mg L^{-1} and 1.00 mg L^{-1} concentrations. The vials were gently shaken and the medium poured into plastic Petri dishes (90 mm x 15 mm) sterilized with formaldehyde vapor in a laminar flow. In order to obtain the stock suspension sand, the other concentrations of each active ingredient were based on the formula C1 x V1 = C2 x V2, where C1 = more concentrated solution; V1 = volume needed for a more concentrated solution; C2 = final concentrated solution; and V2 = desired volume for the final solution.

The day after the culture media had been prepared, 6.0 mm diameter mycelia disks of *M. phaseolin*a, taken from colonies after seven days of growth, were placed in the center of each Petri dish containing substrate amended with the fungicide concentrations. The dishes were sealed with plastic wrap and incubated in a growth chamber at 25 ± 2ºC, for a 12-hour photoperiod, provided by three fluorescent lamps (Osram daylight 40 W), positioned at 50 cm above the dishes.

The mycelial growth measurement was performed with a pachymeter, by measuring the colonies diameter in two perpendicular directions, when the fungal growth in the control treatment reached the dish edge.

A complete randomized experimental design was used, consisting of seven treatments and four replications, with each Petri dish being considered an experimental unit. The experiment was performed twice and the average of two tests was used in the statistical analysis.

The colony diameter measures (mm) were transformed to control (inhibition) percentage and subjected to statistical analysis (fungicide x isolate). The Costat statistical program was used for the logarithmic regression analysis. The concentration to inhibit 50% of the fungus mycelial growth (IC_{50}) in the fungicides tested was calculated from the generated equations.

RESULTS AND DISCUSSION

The monitoring of fungus sensitivity to fungicides is important for maximizing its control efficiency. The IC_{50} is specific and constant for a particular chemical agent and to a particular pathogen. The substance is fungicidal in a low concentration, and a low IC_{50} value represents a high fungicidal action or fungicidal power (Reis et al. 2007).

The fungus sensitivity to a fungicide, or a chemical fungitoxicity, is measured by parameters such as the IC_{50} (concentration that inhibits 50% of the mycelium growth and spore germination) (Sharvelle 1961, Torgeson 1967, Edgington et al. 1971, Reis et al. 2010).

By measuring the *M. phaseolina* colony diameter in each treatment, the IC_{50} values (Table 2) were calculated. The coefficients of determination ranged 0.87-0.98. The sensitivity of a fungus to a toxic substance (fungicide), or the measurement of the chemical toxicity to a fungus, is expressed by ED_{50} (effective dose), EC_{50} (effective concentration) or IC_{50} (inhibitory concentration). The fungicides that showed the highest inhibition level (IC_{50} below

Table 2. Fungicide, regression equation, coefficient of determination (R^2), sensitivity and 50% inhibitory concentration of mycelium growth (IC_{50}) of *Macrophomina phaseolina* (Passo Fundo, RS, 2011).

Fungicide	Regression equation*	R^2	IC_{50}**	S(1)
		%	mg L^{-1}	
Carbendazim	y = -10.9 Ln(x) + 34.18	0.87	0.23	HS
Metalaxyl	y = -0.15 Ln(x) + 99.52	0.83	> 40	I
Fluquinconazol	y = -1.73 Ln(x) + 94.90	0.80	> 40	I
Iprodione	y = -13.1 Ln(x) + 51.60	0.94	1.13	MS
Penflufen + trifloxystrobin	y = -10.8 Ln(x) + 47.66	0.98	0.81	HS
Pyraclostrobin	y = -9.82 Ln(x) + 66.87	0.91	5.57	MS
Thiram	y = -2.18 Ln(x) + 93.86	0.60	> 40	I
Tolyfluanid	y = -6.60 Ln(x) + 80.50	0.80	> 40	I

* y = percentage of mycelial growth inhibition; x = fungicide concentration. ** Calculated by the concentration equation (mg L^{-1}). (1) Sensitivity of *Macrophomina phaseolina* to fungicide: high sensitivity (HS), moderate sensitivity (MS), low sensitivity (LS), insensitive (I). Average of two experiments.

1.00 mg L^{-1}) were carbendazim and penflufen + trifloxystrobin (testing fungicide) (Figure 1).

In the two experiments, an average concentration of 0.23 mg L^{-1} for the active ingredient of the carbendazim IC_{50} value was observed. This fungicide proved to be the most fungitoxic to the *M. phaseolina* isolate (Table 2 and Figure 3). The penflufen + trifloxystrobin mixture was also efficient, showing a CI_{50} of 0.81 mg L^{-1}. For both fungicides, this isolate was considered highly sensitive.

Edgington et al. (1971) proposed the following criteria to frame a fungicidal substance, concerning fungitoxicity: ED_{50} < 1 mg L^{-1} = highly fungitoxic, ED_{50} of 1-50 mg L^{-1} = moderately fungitoxic and ED_{50} > 50 mg L^{-1} = non-toxic. The same authors reported an IC_{50} of 1.13 mg L^{-1} for the iprodione fungicide, which is considered a moderately fungitoxic chemical.

The IC_{50} represents the chemical concentration to inhibit (or control) 50% of the mycelial growth (mm) or potentially viable spores germination (%), lesions (leaf spots) number cm^{-2} and uredia density cm^{-2}.

Due to its genetics, a fungus can be sensitive or not to a given molecule. If a fungus is sensitive to a fungicide, it displays fungitoxicity, otherwise, it is non-toxic. If the fungicide shows no fungitoxicity, the fungus is then considered insensitive (Reis et al. 2007). Not all chemicals are toxic to fungi and a fungicide does not control all fungi (Sharvelle 1961, Torgeson 1967, Edgington et al. 1971, Reis et al. 2010).

The active ingredients fluquinconazole, metalaxyl, thiram and tolyfluanid (Table 2) showed IC_{50} values higher than 40.00 mg L^{-1}, indicating the isolate insensitivity. For the active ingredient pyraclostrobin, the pathogen was considered moderately sensitive, with IC_{50} value of 5.57 mg L^{-1} and coefficient of determination of 0.91.

According to Edginton et al. (1971), fluquinconazole, metalaxyl, tolyfluanid and thiram can be classified as non-toxic active ingredients to the *M. phaseolina* isolate. None of the fungicide treatments inhibited 100% of the fungal mycelium growth (Figures 1 and 2).

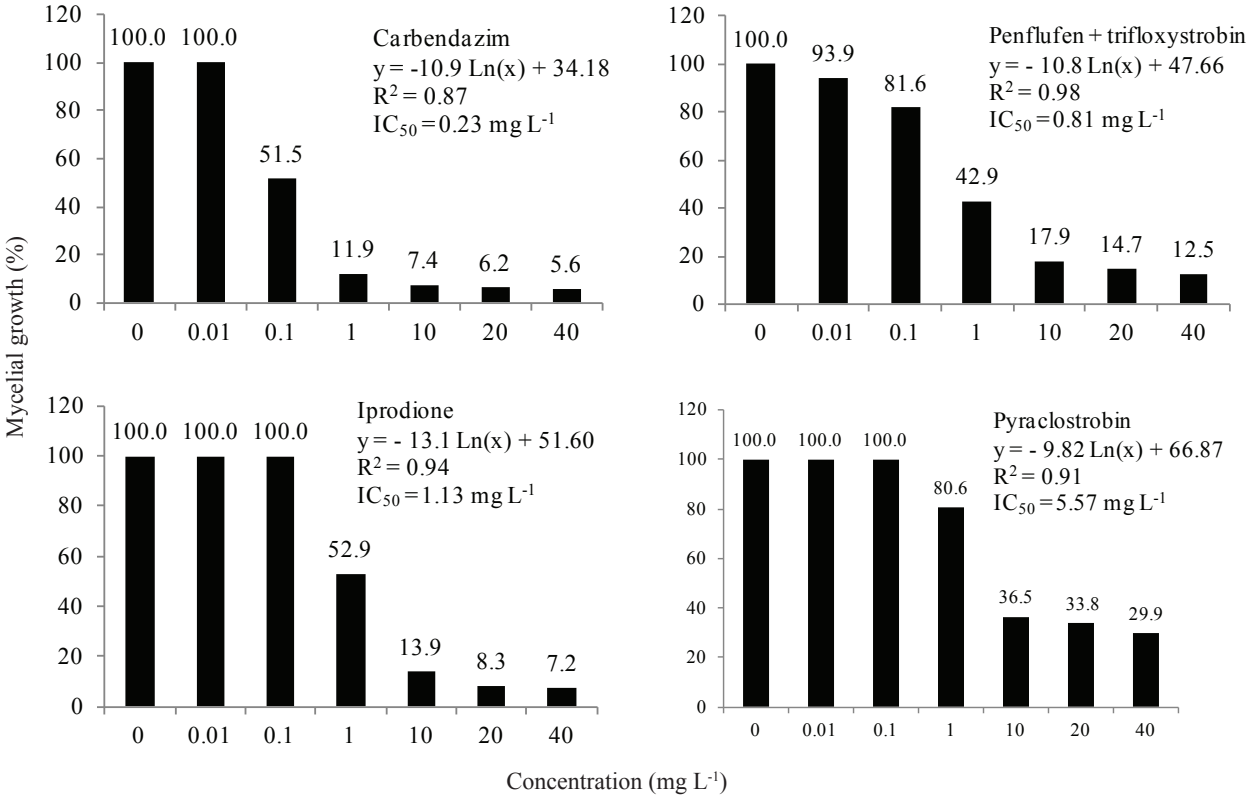

Figure 1. *In vitro* mycelial growth of *Macrophomina phaseolina* isolates, in seven concentrations (mg L^{-1} a.i.) of carbendazim, penflufen + trifloxystrobin, iprodione and pyraclostrobin (Passo Fundo, RS, 2011). y = mycelial growth; x = fungicide concentration; IC_{50} = 50% inhibitory concentration for mycelial growth.

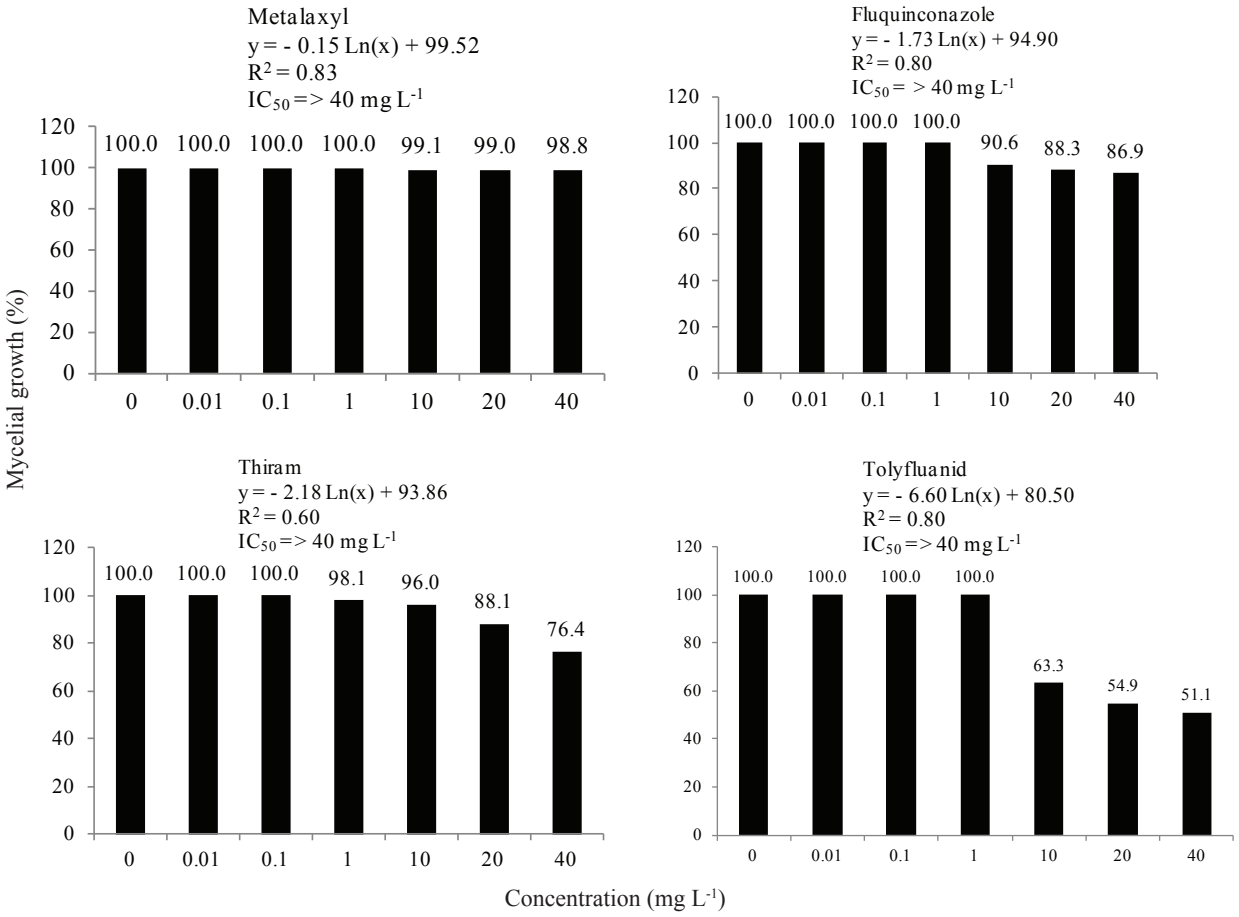

Figure 2. *In vitro* mycelial growth of *Macrophomina phaseolina* isolates, in seven concentrations (mg L^{-1} a.i.) of metalaxyl, fluquinconazole, thiram and tolyfluanid (Passo Fundo, RS, 2011). y = mycelial growth; x = fungicide concentration; IC_{50} = 50% inhibitory concentration for mycelial growth.

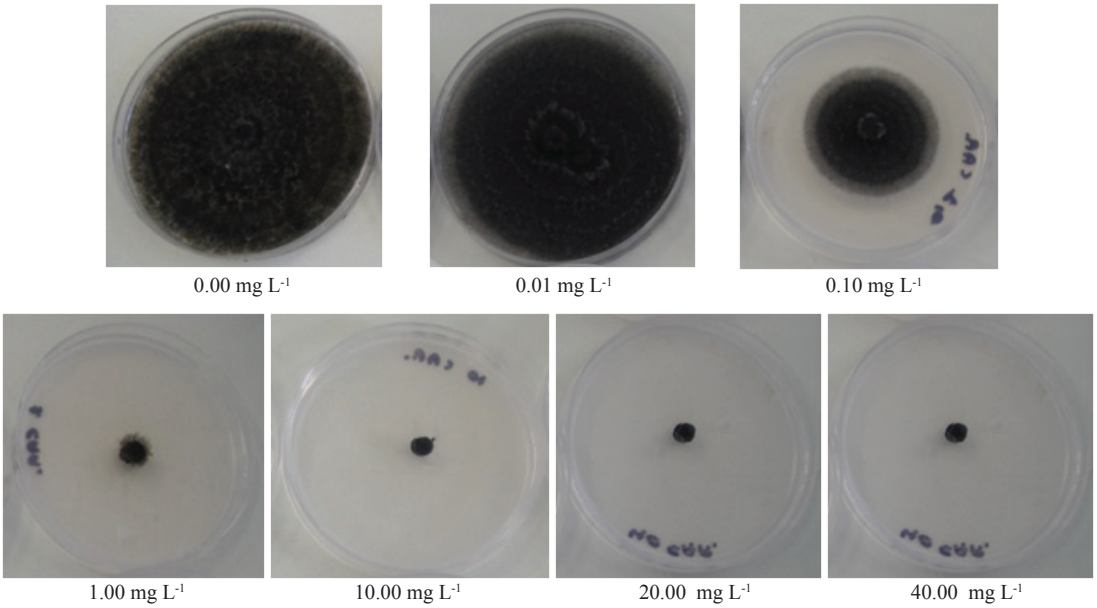

Figure 3. Mycelial growth of *Macrophomina phaseolina* soybean isolate, in a culture medium supplemented with different concentrations of carbendazim (Passo Fundo, RS, 2011).

Only a few studies have reported the sensitivity of this fungus to fungicides. Menten et al. (1976), studying the effect of three fungicides on the mycelium growth of *M. phaseolina*, concluded that benomyl promoted a greater mycelial growth inhibition than pentachloronitrobenzene (PCNB) and carboxin. Benomyl, a benzimidazol compound, belongs to the same chemical group of carbendazim.

In a study conducted by Braga et al. (2003), using benomyl and thiophanate methyl, in a *Vigna unguiculata* (L.) Walp. seed treatment, the *M. phaseolina* transmission did not differ between the two fungicides. In the present study, the IC_{50} of the active ingredient carbendazimcan showed to be a viable alternative for controlling the fungus in soybean seeds. However, the effective control of *M. phaseolina* by seed treatment with fungicides has not been considered.

Just a few scientific studies have reported the fungicides performance for controlling this fungus. The first step in the search for promising products which chemically control this pathogen is to identify the fungus isolates sensitivity to fungicides available in the market. The IC_{50} values for the fungicides were different in magnitude, showing chemical toxicity to non-toxicity to the isolate.

CONCLUSIONS

1. The active ingredients carbendazim and penflufen + trifloxystrobin were the most powerful ones to control *M. phaseolina*, or the most efficient in soybean seed treatments.
2. The *M. phaseolina* isolate showed insensitivity to the active ingredients fluquinconazole, metalaxyl, thiram and tolyfluanida.

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