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Sensitivity of *Colletotrichum lindemuthianum* from green beans to fungicides and race determination of isolates from State of São Paulo, Brazil

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This study determined the physiological races of *Colletotrichum lindemuthianum* isolates, causal agent of anthracnose, collected in green bean producing regions, and assessed the *in vitro* and *in vivo* sensitivity of isolates to fungicides. Physiological races of isolates were determined by inoculation of bean differential cultivars under controlled conditions. *In vitro* sensitivity of colony growth and conidial germination were evaluated for carbendazim, chlorothalonil, copper oxide, mancozeb, pyraclostrobin, thiophanate-methyl and for the mixtures of mancozeb + copper oxychloride, metiram + pyraclostrobin and thiophanate-methyl + chlorothalonil at concentrations of 1, 10, 100 and 1000 µg/mL on PDA medium. *In vivo* sensitivity was determined in detached primary leaves of green beans previously treated with the same fungicides (commercial doses) recommended for the crop, and then inoculated with conidial suspensions of *C. lindemuthianum*. *C. lindemuthianum* isolates were identified as belonging to races 65 and 81. Treatments with pyraclostrobin and the mixture metiram + pyraclostrobin were the most effective in inhibiting the colony growth and conidial germination *in vitro*, a result also observed for the *in vivo* experiments, where these chemicals were the most effective in controlling the green bean anthracnose.

Key words: Chemical control, *Phaseolus vulgaris*, anthracnose, physiological race, snap bean.

INTRODUCTION

Anthracnose, caused by *Colletrotrichum lindemuthianum*, is one of the major diseases of green bean, occurring in almost all producing countries, including Brazil. The disease affects leaves and pods of the plants, reducing productivity and the product quality for commercialization, being the cultivation of susceptible cultivars in regions with mild temperatures and high humidity one of the main factors for this wide distribution (Dalla Pria et al., 2003).

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Use of resistant cultivars and chemical control are essential for the efficient control of anthracnose (Ghini and Kimati, 2000). The knowledge of the physiological races of *C. lindemuthianum* that occurs on green beans and the sensitivity of isolates to fungicides are fundamental for the adoption of effective control measures, whether by the use of resistant cultivars to prevalent races of the pathogen in a region or for the chemical control in susceptible cultivars with appropriate fungicides.

Early studies in Brazil about the races of *C. lindemuthianum* were made during the 60's and 70's, but due to high pathogenic variability of the pathogen and the small number of differential cultivars used, the results were not reliable, as well as the use of different systems for naming the races caused difficulties to measure the variability in the fungus pathogenicity (Paradela Filho et al., 1991; Rodriguez-Guerra et al., 2003). After the work of Pastor-Corrales (1991), using 12 differential bean cultivars to study the pathogenic variability of *C. lindemuthianum* isolates, there was the standardization and creation of a binary scale for symptoms assessment, according to anthracnose severity, which provided the description of new races and breeding of new sources of resistance. Several studies have identified the occurrence of races of *C. lindemuthianum* in different regions of Brazil (Somavilla and Prestes, 1999; Thomazella et al., 2002; Bonett et al., 2008), but without specifying the origin of the isolates according to host (common beans or green beans).

Chemical control of anthracnose with fungicides have shown satisfactory results, resulting in high grain yield in common bean (Conner et al., 2004; Garcia et al., 2007; Gillard et al., 2012). In Brazil, there are more than 100 chemicals registered for the control of anthracnose in common bean, however, for green beans, only seven fungicides, from four different active ingredients (copper oxide, mancozeb, mancozeb + copper oxycloride and thiophanate-methil) are registered for use (Agrofit, 2015). Some chemicals used to control the anthracnose of common beans cannot be used on green bean, due to problems of residues. *C. lindemuthianum* isolates from common beans have shown a variation in the sensitivity of mycelial growth for benzimidazole fungicides (benomyl, carbendazim and thiophanate-methyl) (Maringoni and Barros, 2002; Sartorato, 2007; Sartori and Maringoni, 2007), but there is no information about the sensitivity of isolates from green beans.

The knowledge of physiological races and the sensitivity of *C. lindemuthianum* isolates to fungicides are essential for the efficient management of the anthracnose of green bean. On this study, the physiological races of 12 monosporic isolates of *C. lindemuthianum* from green bean producing regions of State of São Paulo, Brazil, were identified, as well the *in vitro* and *in vivo* sensitivity of isolates to fungicides recommended for the crop were determined.

MATERIALS AND METHODS

Cultures and growth conditions

Pods of green beans naturally infected by *C. lindemuthianum* were collected from producing regions of State of São Paulo, Brazil. Small portions of conidia present in the lesions were transferred to potato dextrose agar medium (PDA) (Acumedia, Baltimore, USA), followed by incubation in the dark (25°C/10 days). Isolates 3149, 3151, 3152, 3153 and 3158 (collected in Botucatu), 3148 (Campinas), 3154 (Itatiba), 3147 (Jaú), 3150 (Morungaba), 3156 (São Manuel), 3155 (São Paulo) and 3157 (Vinhedo) were purified and monosporic cultures obtained and preserved according to Castellani's modified method (Dhingra and Sinclair, 1995). All isolates had typical cultural and morphological characteristics of *C. lindemuthianum* according to Schwartz (1991). For all experiments, inoculum was produced on PDA in the dark (25°C/10 days). For the *in vitro* sensitivity of conidial germination experiment, inoculum was produced on oatmeal agar medium (60 g oat meal, 12 g agar and 1.000 mL distilled water) at same conditions, but for 15 days.

In vitro sensitivity of *C. lindemuthianum* isolates to fungicides

Colony growth sensitivity of all isolates were assessed to commercial fungicides carbendazim, chlorothalonil, copper oxide, mancozeb, pyraclostrobin, tiophanate-methyl and to the mixtures mancozeb + copper oxychloride (57% + 43%), metiram + pyraclostrobin (92% + 8%) and chlorothalonil + thiophanate-methyl (71% + 29%) at 1, 10, 100 and 1000 µg/mL in 90 × 16 mm Petri dishes with PDA medium. PDA medium without fungicides was used as control. Mycelial discs (7 mm) were transferred to the Petri dishes with the treatments and plates were incubated in the dark (25°C/10 days). The average diameter of the colonies (mm) was assessed and the percentage of inhibition calculated based in the control treatment growth. Experimental design was completely randomized with four replications for each fungicide concentration and for each isolate. The effective dose causing 50% inhibition of mycelial growth (ED_{50}) was determined according to Sartori and Maringoni (2007), and numerical index for each range of ED_{50} were assigned: index 1 (ED_{50} < 1 µg/mL), index 2 (ED_{50} from 1 to 10 µg/mL), index 3 (ED_{50} from 10 to 100 µg/mL), index 4 (ED_{50} from 100 to 1000 µg/mL) and index 5 (ED_{50} > 1000 µg/mL).

Conidial germination sensitivity was evaluated for isolates 3147, 3150, 3155 and 3158 that showed sensitivity in the colony growth experiment to mancozeb and to the chlorothalonil + tiophanate-methyl mixture. PDA preparation, fungicides and concentrations were the same used for the assessment of colony growth sensitivity. Suspensions were standardized to 5×10^5 conidia/mL (Maringoni and Barros, 2002) and 100 µL of each suspension were distributed on the surface of the media with fungicides followed by incubation in the dark (25°C/24 h). Mycelial discs (1.5 cm) were transferred to microscope slides and stained with lactophenol cotton blue to paralyze the fungal growth. For each isolate and fungicide concentration the germination of 100 conidia were assessed per disc. Experimental design was completely randomized with four replications and each plot represented by a mycelial disc. With the results the effective dose causing 50% inhibition of conidial germination (ED_{50}) was determinated according to the numerical index previously described.

In vivo sensitivity of *C. lindemuthianum* isolates to fungicides

Green beans plants cv. Itatiba II were cultivated in 2 L pots with substrate (mixture of soil, weathered cattle manure and sand (1:1:1), plus 0.6 kg of ammonium sulfate, 17 kg of superphosphate, 0.6 kg of potassium chloride and 0.8 kg of lime for every m^3 of the

Table 1. Effective dose causing 50% inhibition of mycelial growth (ED_{50}) for *Colletotrichum lindemuthianum* isolates from green beans to fungicides.

Fungicides	Isolate											
	3147	3148	3149	3150	3151	3152	3153	3154	3155	3156	3157	3158
Carbendazim	5	5	5	5	5	5	5	5	5	4	5	5
Chlorothalonil	4	5	4	3	4	4	3	3	3	3	4	4
Chlorothalonil + thiop.-methyl	4	5	4	4	4	4	3	4	4	4	5	4
Copper oxide	5	5	5	5	5	5	5	5	5	5	5	5
Mancozeb	5	5	5	5	5	5	4	5	4	5	5	5
Mancozeb + copper oxychloride	5	5	5	5	5	5	5	5	5	5	5	5
Metiram + pyraclostrobin	3	4	4	3	4	4	4	4	4	4	4	4
Pyraclostrobin	2	2	2	2	2	2	2	2	2	2	2	2
Thiophanate-methyl	5	5	5	5	5	5	5	5	5	5	5	5

Numerical index from 1 to 5 correspond to different ranges of ED_{50}: index 1 - ED_{50} < 1 µg/mL; index 2 - ED_{50} from 1 to 10 µg/mL; index 3 - ED_{50} from 10 to 100 µg/mL; index 4 - ED_{50} from 100 to 1000 µg/mL and index 5 - ED_{50} > 1000 µg/mL.

mixture) under greenhouse conditions (20-28°C/70-90% RU) till the phenological growth stage V2. The primary leaves were collected, immersed in a solution of 10% Tween 80 (10 s), and then immersed, separately, in suspensions of commercial fungicides carbendazim (1 mL/L), chlorothalonil (1.5 g/L), copper oxide (1.72 g/L), mancozeb (3.20 g/L), mancozeb + copper oxychloride (0.88 + 0.66 g/L), metiram + pyraclostrobin (1,65 + 0.15 g/L), pyraclostrobin (0.15 mL/L), thiophanate-methyl (1.40 g/L) and chlorothalonil + thiophanate-methyl (1.75 + 0.70 g/L) for 5 s. The leaves were transferred in Petri dishes (150 × 15 mm) containing two paper filters wetted with distilled water and sprayed with conidial suspensions (10^6 conidia/mL) of each isolate, after 24 h of the fungicide treatment (Gulart, 2009). Petri dishes containing two primary leaves each were kept in the dark for 24 h and incubated in BOD (20°C/7 days) under a 12 h photoperiod (2400 Lux). Control treatment consisted of leaves immersed in water and inoculated with conidial suspensions. Absolute control consisted of leaves immersed in water without inoculation. Disease severity on leaves was determined seven days after inoculation according to the diagrammatic scale proposed by Dalla Pria et al. (2003). Experimental design was completely randomized, with three replicates for each isolate and fungicide. Data were subjected to variance analysis and means were compared by Scott-Knott's test at 5% probability.

Determination of physiological races of *C. lindemuthianum* isolates

Seedlings of bean differential cultivars AB 136, Cornell 49-242, G 2333, Kaboon, Mexico 222, Michelite, Michigan Dark Red Kidney, Perry Marrow, Pi 207262, TO, TU and Widusa were obtained under greenhouse conditions in plastic trays of 84 cells containing sterile vermiculite. The aerial part of five seedlings of each cultivar were inoculated by spraying with a suspension (10^6 conidia/mL) of each isolate, 10 days after emergence, according to Pastor-Corrales (1991) and Carbonell et al. (1999). One plant of Pérola cultivar, susceptible to most races of *C. lindemuthianum*, was used as a negative control for each differential series. Seedlings were kept in a climatic room (23°C/90% RU) under a 12 h photoperiod (2400 Lux).

The disease severity was evaluated 10 days after inoculation based on disease scale from 1 to 9, with grades from 1 to 3 representing resistant genotypes, and grades above 3, susceptible genotypes (Pastor-Corrales, 1991; Gonçalves-Vidgal et al., 2007).

RESULTS

In vitro sensitivity of *C. lindemuthianum* isolates to fungicides

Results of colony growth and conidial germination *in vitro* sensitivity of *C. lindemuthianum* isolates to fungicides are described in Tables 1 and 2, respectively. All isolates showed low sensitivity on colony growth and conidial germination (ED_{50} > 1000 µg/mL) to thiophanate-methyl. For chlorothalonil and thiophanate-methyl + chlorothalonil, 50% and 75% of isolates had ED_{50} between 100 and 1000 µg/mL for colony growth, respectively. For conidial germination, 100% of the isolates showed ED_{50} between 10 and 100 µg/mL to chlorothalonil + thiophanate-methyl, while chlorothalonil was the most effective fungicide for inhibition of conidial germination of isolates (100% of isolates with ED_{50} < 1 µg/mL). It was also observed a low sensitivity to carbendazim (ED_{50} > 1000 µg/mL) to 100% (conidial germination) and 91.7% (colony growth) of the isolates.

Regarding to mancozeb and mancozeb + copper oxychloride, 83.4% and 100% of isolates had an ED_{50} higher than 1000 µg/mL for colony growth, respectively, while 100% of the isolates showed ED_{50} between 10 and 100 µg/mL for conidial germination for these chemicals. All isolates evaluated were sensitive to pyraclostrobin and metiram + pyraclostrobin (ED_{50} from 1 to 10 µg/mL) for conidial germination. For colony growth, the sensitivity was reduced in the mixture metiram + pyraclostrobin (83.4% of isolates with ED_{50} from 100 to 1000 µg/mL) in relation to pyraclostrobin (100% of isolates with ED_{50} from 1 to 10 µg/mL). The isolates did not show sensitivity to copper oxide with an ED_{50} higher than 1000 µg/mL for colony growth and conidial germination.

Table 2. Effective dose causing 50% inhibition of conidial germination (ED_{50}) for *C. lindemuthianum* isolates from green beans to fungicides.

Fungicides	Isolate			
	3147	3150	3155	3158
Carbendazim	5	5	5	5
Chlorothalonil	1	1	1	1
Chlorothalonil + thiop.-methyl	3	3	3	3
Copper oxide	5	5	5	5
Mancozeb	3	3	3	3
Mancozeb + *copper* oxychloride	3	3	3	3
Metiram + pyraclostrobin	2	2	2	2
Pyraclostrobin	2	2	2	2
Thiophanate-methyl	5	5	5	5

Numerical index from 1 to 5 correspond to different ranges of ED_{50}: index 1 - ED_{50} < 1 µg/mL; index 2 - ED_{50} from 1 to 10 µg/mL; index 3 - ED_{50} from 10 to 100 µg/mL; index 4 - ED_{50} from 100 to 1000 µg/mL and index 5 - ED_{50} > 1000 µg/mL.

Table 3. Anthracnose severity in detached primary leaves of green bean cv. Itatiba II treated with fungicides and inoculated with *C. lindemuthianum* isolates.

Fungicide	Isolate			
	3147	3150	3155	3158
Control (water)	71.7[a]*	100.0[a]**	100.0 [a]	100.0[a]
Carbendazim	66.7[a]	41.7[b]	51.7 [b]	46.7[c]
Chlorothalonil	4.0[c]	1.7[c]	1.7 [e]	1.7[e]
Chlorothalonil + thiop.-methyl	8.7[c]	6.0[c]	9.3 [d]	8.0[d]
Copper oxide	31.7[b]	40.0[b]	35.0 [c]	40.0[c]
Mancozeb	1.7[c]	2.7[c]	1.7 e	3.0[e]
Mancozeb + *copper* oxychloride	4.0[c]	2.7[c]	2.0 e	5.0[d]
Metiram + pyraclostrobin	0[f]	0[f]	0[f]	0[f]
Pyraclostrobin	0[f]	0[f]	0[f]	0[f]
Thiophanate-methyl	41.7[b]	43.3[b]	43.3[b]	55.0[b]
V.C.%	17.6	17.0	10.7	9.6

*Data transformed in arcsen√x/100. **Means followed by same letter are not significantly different at P<0.05 probability using Scott-Knott's test.

In vivo sensitivity of *C. lindemuthianum* isolates to fungicides

Results of *in vivo* sensitivity of *C. lindemuthianum* isolates to fungicides are described in Table 3, and were very similar to those obtained from the *in vitro* experiments. Pyraclostrobin and metiram + pyraclostrobin were the most effective fungicides for the control of anthracnose on green beans leaves, independent of the *C. lindemuthianum* isolate evaluated, showing complete absence of symptoms in the inoculated leaves.

The treatments with chlorothalonil, mancozeb and the mixtures mancozeb+ copper oxychloride and chlorothalonil + thiophanate-methyl were also effective in the reduction of anthracnose severity on green beans leaves. However, the fungicides from the group of benzimidazole (carbendazim and thiophanate-methyl) and copper oxide did not controlled the disease on the leaves, similar results to those obtained from *in vitro* studies.

Determination of physiological races of *C. lindemuthianum* isolates

Isolates 3149, 3151, 3152, 3158, 3147, 3148, 3150 and 3157 of *C. lindemuthianum* were identified as race 65, while isolates 3153, 3154, 3155 and 3135 were identified as race 81 (Table 4).

DISCUSSION

The variation in colony growth sensitivity of *C. lindemuthianum* isolates from common bean to

Table 4. Reaction of bean differential cultivars inoculated with *C. lindemuthianum* isolates from green beans from various locations of São Paulo State.

Differential cultivar	Binary value	Isolate											
		3147	**3148**	**3149**	**3150**	**3151**	**3152**	**3153**	**3154**	**3155**	3156	3157	3158
Michelite	1	S	S	S	S	S	S	S	S	S	S	S	S
Michigan Dark Red Kidney	2	R	R	R	R	R	R	R	R	R	R	R	R
Perry Marrow	4	R	R	R	R	R	R	R	R	R	R	R	R
Cornell 49-242	8	R	R	R	R	R	R	R	R	R	R	R	R
Widusa	16	R	R	R	R	R	R	R	R	R	R	R	R
Kaboon	32	R	R	R	R	R	R	R	R	R	R	R	R
México 222	64	S	S	S	S	S	S	S	S	S	S	S	S
Pi 207262	128	R	R	R	R	R	R	R	R	R	R	R	R
TO	256	R	R	R	R	R	R	R	R	R	R	R	R
TU	512	R	R	R	R	R	R	R	R	R	R	R	R
AB 136	1024	R	R	R	R	R	R	R	R	R	R	R	R
G 2333	2048	R	R	R	R	R	R	R	R	R	R	R	R
Race		65	65	65	65	65	65	81	81	81	81	65	65

benzemidazole fungicides (benomyl, carbendazim and thiophanate-methyl) has been reported in several studies in Brazil with ED_{50} ranging from 1 to 1000 µg/mL (Maringoni and Barros, 2002; Sartorato, 2007; Sartori and Maringoni, 2007). On this study we detected an low sensitivity of colony growth of *C. lindemuthianum* isolates from green beans to benzimidazoles fungicides (carbendazim and thiophanate-methyl) (Table 1). The low sensitivity of conidial germination from *C. lindemuthianum* isolates evaluated on this study to carbendazim and thiophanate-methyl agrees with Maringoni and Barros (2002), where isolates from common beans were also resistant to these fungicides.

C. lindemuthianum isolates from common beans also exhibit variation in colony growth sensitivity to chlorothalonil with ED_{50} ranging from 10 to 800 µg/mL (Rava et al., 1998; Maringoni and Barros, 2002), results similar to the obtained in this study for green beans isolates. Most isolates here evaluated showed an ED_{50} from 100 to 1000 µg/mL for colony growth to chlorothalonil + thiophanate-methyl, results discrepant to those obtained by Balardin and Rodrigues (1995) and Sartori and Maringoni (2007), who reported inhibition of *C. lindemuthianum* isolates from common beans to concentrations ranging from 10 to 100 µg/mL. Regarding conidial germination, chlorothalonil was the most effective fungicide (ED_{50} < 1 µg/mL), while thiophanate-methyl did not show a satisfactory result (ED_{50} > 1000 µg/mL). These results show that the best performance of the mixture chlorothalonil + thiophanate methyl (ED_{50} from 10 to 100 µg/mL) in comparison with thiophanate-methyl alone is due to the chlorothalonil activity, showing the absence of a synergistic action between these fungicides.

C. lindemuthianum isolates evaluated in this study showed an ED_{50} for mancozeb and the mixture mancozeb + copper oxychloride higher to those described by Rava et al. (1998) for isolates from common beans, indicating a lower sensitivity of these isolates to these chemicals. No information about the sensitivity of *C. lindemuthianum* isolates to copper oxide was found, although Tsai et al. (2006) evaluated isolates of *C. gloesporioides* and *C. musae* from fruit species and found and ED_{50} ranging from 10 to 100 µg/mL for cupric fungicides, values lower to those found on this study.

ED_{50} values for the colony growth to pyraclostrobin found on this study were lower to those reported by Sartorato (2007) for *C. lindemuthianum* isolates from common beans (ED_{50} of 187.5 µg/mL) and close to the results obtained by Sartori and Maringoni (2007) to trifloxystrobin, a strobilurin from the same group of pyraclostrobin. However, colony growth sensitivity

of *C. lindemuthianum* isolates from green beans was reduced for the mixture metiram + pyraclostrobin, which most of the isolates had and ED_{50} ranging from 100 to 1000 µg/mL, concentration higher than that reported by Tsai et al. (2006) for *C. gloesporioides* and *C. musae* (ED_{50} from 10 to 100 µg/mL). There is no information about conidial germination sensitivity from *C. lindemuthianum* isolates to pyraclostrobin and metiram + pyraclostrobin, however, there is information about other fungi, such as *Cylindrocladium candelabrum* on eucalyptus (Ferreira et al., 2006) and *Cercospora sojina* on soybean (Zhang et al., 2012), demonstrating the sensitivity of conidial germination of these fungi to strobilurins.

Pyraclostrobin was also the most effective fungicide for the control of anthracnose on green bean leaves, a result that agrees with Conner et al. (2004) and Gillard et al. (2012) for white and common beans, respectively. The high sensitivity of isolates here evaluated to metiram + pyraclostrobin may have occurred due probably to the sensitivity showed by them to the pyraclostrobin. There are no reports of *C. lindemuthianum* sensibility to metiram and copper oxide.

The disease severity reduction found on this study for the mixture chlorothalonil + thiophanate-methyl, compared to thiophanate-methyl alone, demonstrates the sensitivity of *C. lindemuthianum* isolates to chlorothalonil and the low sensitivity to benzimidazole fungicides, results also observed by Garcia et al. (2007) in common beans.

According to Bashir et al. (1985), benomyl and chlorothalonil were effective for the control of anthracnose on mung bean; results similar to those obtained in this study to chlorothalonil, but differed for thiophanate-methyl, fungicide with mode of action similar to benomyl. Castro et al. (1991) also reported the efficiency of chlorothalonil for the control of anthracnose in common beans.

The efficiency of mancozeb and mancozeb + copper oxychloride observed on this study in reducing the severity of anthracnose on green bean leaves also agrees with Castro et al. (1991), who reported a disease severity reduction in common beans treated with these chemicals. The authors also demonstrated the effectiveness of carbendazim in controlling the anthracnose in common beans, result not observed on this study with green bean isolates.

Studies with *C. lindemuthianum* isolates from common beans also identified the prevalence of races 65 and 81 in Brazil (Somavilla and Prestes, 1999; Carbonell et al., 1999; Thomazella et al., 2002). Until now there was no information about the races of *C. lindemuthianum* that occur on green beans demonstrating that genotypes with resistance to races 65 and 81 of *C. lindemuthianum* should be used in genetic breeding programs of green beans, aiming the incorporation of resistance genes to anthracnose in susceptible cultivars.

Conflict of Interest

The authors have not declared any conflict of interest.

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