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ORIGINAL ARTICLE

Detection and chemical control of *Cercospora sojina* infecting soybean seed in Argentina

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Abstract
Frogeye leaf spot (FLS) of soybean caused by *Cercospora sojina* Hara is an important disease in the Argentine Pampas region. The primary inoculum sources of the disease are infected seeds and plant debris. Thus, use of pathogen-free or fungicide-treated seeds is crucial to prevent the introduction and further spread of the disease. The aims of this research were i) to quantify *C. sojina* incidence in harvested soybean seeds, ii) to compare media-based incubation methods for detection of the pathogen in seeds, and iii) to evaluate the effect of fungicide seed treatments in reducing FLS incidence. Among eight different detection methods evaluated, no difference in pathogen incidence was found, but the water restriction blotter method should be recommended as it is more time and cost-effective. Out of 13 different fungicide seed treatment combinations in three independent experiments, benzimidazole seed treatments resulted in zero FLS incidence. Any other of the tested fungicidal active ingredients either did not control the disease or did so poorly only when initial infection levels were low. Treating seeds with benzimidazole fungicides may help to reduce FLS incidence during initial soybean field stands when using infected seed lots.

Keywords Frogeye leaf spot · *Glycine max* · Seed health testing · Fungicide treatments

Introduction

Frogeye leaf spot (FLS) of soybean [*Glycine max* (L.) Merr.], caused by *Cercospora sojina* Hara is of common occurrence in most soybean-growing countries (Phillips 1999). The pathogen primarily infects soybean foliage though it can also infect seeds, pods, and stems (Phillips 1999).

Severe FLS epidemics were reported during the 1998–1999 growing season in the North West region of Argentina (Ploper et al. 2000). Outbreaks were also observed in the provinces of Córdoba, Santa Fe and Buenos Aires from 2005 to 2008, and the disease was found in the Pampas Region of the country between those years too, though at low prevalence (5–25%). During the 2008–2009 growing season, FLS spread rapidly throughout most soybean growing areas of the Pampas Region, being found in almost all soybean varieties of maturity group III, IV and V, which are susceptible to FLS (Carmona et al. 2009). Economic losses due to FLS estimated during the 2009/2010 soybean season were estimated at about 2000 million dollars (Carmona 2011, Carmona et al. 2015).

One of the most important sources of inoculum for *C. sojina* is soybean residue where the pathogen survives during its saprophytic stage during the off-season. Disease surveys conducted from November to December 2009 in Argentina (Carmona et al. 2009) identified FLS in as early as the vegetative stages (V6-V8) in many fields under conservation tillage and monoculture, which are reported to favor disease development (Carmona et al. 2009; Scandiani et al. 2012). Nevertheless, the disease has also been found in fields never planted to soybean or under soybean rotation, indicating that the pathogen was likely introduced *via* infected seeds. Seedborne inoculum is likely the cause of FLS epidemics spread and the introduction of new races in the Argentine Pampas region. The fungus can penetrate the pod walls and

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infect the developing seeds (Singh and Sinclair 1985; Phillips 1999; Roy et al. 2001). Infected seeds may show discoloration of the seed coat and midsize gray to brown blotches, which are the typical symptoms of the disease on seeds (Bisht and Sinclair 1985).

Although *C. sojina* has been detected on/in soybean seed (Lehman 1934), it has not been quantified in mature seed from natural epidemics. In addition, knowledge of the impact of seed-borne infection on FLS development and the effectiveness of fungicide seed treatments in seed health are limited. The objectives of this study were: a) to quantify the incidence of *C. sojina* in soybean seeds; b) to compare different methods for detection of the pathogen in seeds and c) to evaluate different fungicide seed treatments in the control of *C. sojina*. Preliminary findings on these areas were published elsewhere (Scandiani et al. 2009a; Scandiani et al. 2009b).

Materials and methods

Fungal incidence in field samples

Seventy-eight soybean seed samples (1 kg) were obtained from commercial soybean fields displaying FLS foliar symptoms. Samples were collected at physiological maturity during the 2009/2010 growing season from fields located in the south of the Santa Fe province and in the north of the Buenos Aires province, Argentina. The samples were assessed for fungal incidence using the water restriction blotter test (sodium chloride at −1,0 MPa osmotic pressure added to the water, Machado et al. 2003). From each sample, a total of 200 seeds (4 replications of 50 seeds each) were surface-disinfected for 1 min in a 1% sodium hypochlorite (NaOCl) solution, followed by rinsing twice in sterile distilled water. The seeds were plated on trays (16 × 20 × 5 cm, 50 seeds per tray) and incubated at 25 ± 1 °C under alternating periods of 12-h fluorescent cool daylight (Osram 18 W/765) for 7 days (Leach 1963). The seeds were then examined under a stereomicroscope at 40× magnification and the presence of conidiophores and conidia of *C. sojina* were identified at 20 to 40× (Scandiani and Carmona 2009; Scandiani and Luque 2009). To further confirm the identity of the pathogen, the Koch's postulates were performed using plants from healthy seed and from the same cultivars.

Media-based detection methods with incubation

The following methods with incubation of the seeds were used for the analysis: water restriction blotter test [NaCl 11,96 g, deionized water 1 l], carrot soft agar [fresh carrots 100 g, agar 16 g, deionized water 1 l], carrot agar [fresh carrots 600 g, agar 16 g, deionized water 1 l], tomato puree agar [220 mL tomato juice, $CaCO_3$ 3,3 g, agar 16 g, deionized water 1 l], soybean leaves agar [soybean leaves 100 g, agar 16 g, deionized water 1 l], vegetable broth agar [vegetable broth (potato, onion, carrot, leek, tomato, cabbage, spinach, garlic) 15 g, agar 16 g, deionized water 1 l], vegetable broth light agar [vegetable broth (onion, carrot, sweet potato, pumpkin, spinach, leek, tomato, garlic) 3 g, agar 16 g, deionized water 1 l], vegetable broth light agar (no salt) [vegetable broth 5 g, agar 16 g, deionized water 1 l], V8 juice agar [100 mL V8 juice, CO3Ca 1 g, agar 15 g, deionized water 900 mL], potato dextrose agar [potato 200 g, dextrose 20 g, agar 15 g, deionized water 1 l], synthetic nutrient-poor agar [KH_2PO_4 1 g, KNO_3 1 g, $MgSO_47H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, saccharose 0.2 g, agar 20 g, deionized water 1 l] (Scandiani et al. 2009a; Scandiani and Luque 2009).

Three soybean seed samples (each one a replicate of the experiment) with 2.78%, 4.19 and 28.48% *C. sojina* infection according to preliminary analyses were used. The seed samples used in this experiment were infected only with *C. sojina*. All culture media were used for samples 2 and 3, but for sample 1 all media except V8 juice agar, potato dextrose agar and synthetic nutrient agar were used. A total of 400 seeds per sample were disinfected in 1% sodium hypochlorite for 1 min, followed by rinsing twice in sterile distilled water. Seeds were plated on trays (16 × 20 × 5 cm, 50 seeds per tray) and incubated under the same conditions described above. The effect of water restriction method was performed as previously described. The experimental design was a completely randomized block with 8 replications of 50 seeds. The incidence of *C. sojina* was determined as described before.

Incidence (%) values were transformed to $\sqrt{\chi + 1}$ to reduce skewness and stabilize distributional variance. Levene and Shapiro-Wilk tests confirmed the homogeneity and normality of the transformed data, respectively. The analyses were performed with the car (Fox and Weisberg 2011) and stats packages of R (R Core Team 2017), respectively. The data were subjected to analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

Fungicide seed treatments

Three independent replicated experiments were carried out to test different fungicides applied as seed treatments to evaluate their effectiveness in the control of *C. sojina*. Follows details for each experiment.

Experiment 1

Four soybean seed samples (each sample an experiment replicate) were previously selected with 2.0%, 4.0%, 9.44 and 10.5% *C. sojina* infection according to the seed health survey. Four fungicide treatments were

applied to seeds as slurries at commercially recommended doses [rates of fungicides (g a.i./100 L) are given in parentheses]: pyraclostrobin (5) + thiophanate-methyl (45) (Acronis SC, BASF) at 100 mL 100 kg^{-1} seeds, pyraclostrobin (5) + thiophanate-methyl (45) (Acronis SC, BASF) at 150 mL 100 kg^{-1} seeds, fludioxonil (2.5) + metalaxyl (1) (Maxim XL SC, Syngenta) at 100 mL 100 kg^{-1} seeds, and carbendazim (15) + thiram (35) (Ritiram Carb Plus SC, Rizobacter) at 200 mL 100 kg^{-1} seeds. Each treatment was applied to 100 g of seed in a 250-mL Erlenmeyer flask plus water to complete a total volume of 500 mL 100 kg^{-1} of seeds. Slurry-treated seed were allowed to dry for 24 h. Untreated seed, surface disinfested was used as control. Seeds were plated on trays by means of the water restriction blotter test method as previously described. The experimental design was a completely randomized block with 8 replications of 50 seeds. The incidence of *C. sojina*, and the analysis of the data were determined and processed with the same way as described previously.

Experiment 2

Three soybean seed samples (each sample an experiment replicate) were previously selected with 3.35%, 10.72 and 30.87% *C. sojina* infection based on the water restriction blotter assay. Eight fungicide treatments were applied to the seeds as slurries at commercially recommended doses: fludioxonil (2.5) + metalaxyl (3.75) (Apron Maxx RFC SC, Syngenta) at 100 mL 100 kg^{-1} seeds, fludioxonil (2.5) + metalaxyl (3.75) + carbendazim (25) (Apron Maxx RFC SC, Syngenta + Carbendazim, Rizobacter) at 100 + 50 mL 100 kg^{-1} seeds, thiram (35) + carbendazim (15) (Ritiram Carb Plus SC, Rizobacter) at 300 mL 100 kg^{-1} seeds, thiophanate-methyl (45) + pyraclostrobin (5) (Acronis SC, BASF) at 100 mL 100 kg^{-1} seeds, trifloxystrobin (1.38) + metalaxyl (1.1) (Gualicho SC, Bayer) at 350 mL 100 kg^{-1} seeds, carboxin (20) + thiram (20) (Vitavax Flo SC, Arysta Life Science) at 250 mL 100 kg^{-1} seeds, ipconazole (2.5) + metalaxyl (2) (Rancona Dimensión ME, Arysta Life Science) at 100 mL 100 kg-1 seeds, thiram (12.5) (Tiram SC, Rizobacter) at 200 mL 100 kg-1 seeds. The entire procedure was performed as described above.

Experiment 3

Two soybean seed samples (each sample an experiment replicate) were previously selected with 2.45 and 8.79% *C. sojina* infection based on the water restriction blotter assay. Seven fungicide treatments were applied to seeds as slurries at commercially recommended doses: fludioxonil (2.5) + metalaxyl (3.75) (Apron Maxx RFC SC, Syngenta) at 100 mL 100 kg^{-1} seeds, fludioxonil (2.5) + metalaxyl (2) + thiabendazole (15) (Apron Maxx Advanced SC, Syngenta) at 100 mL 100 kg^{-1} seeds, fludioxonil (2.5) + metalaxyl (2) + thiabendazole (15) (Apron Maxx Advanced SC, Syngenta) at 150 mL 100 kg^{-1} seeds, fludioxonil (2.5) + metalaxyl (2) + thiabendazole (15) + thiamethoxam (35) (Cruiser Advanced SC, Syngenta) at 100 mL 100 kg^{-1} seeds, fludioxonil (2.5) + difenoconazole (2.5) + thiamethoxam (26.25) (Cruiser Plus SC, Syngenta) at 300 mL 100 kg^{-1} seeds, thiram (35) + carbendazim (15) (Ritiram Carb Plus SC, Rizobacter) at 200 mL 100 kg^{-1} seeds, trifloxystrobin (1.38) + metalaxyl (1.1) (Gualicho SC, Bayer) at 350 mL 100 kg^{-1} seeds. The entire procedure was performed as described above.

In all the tests carried out, it was evaluated whether the infection with *C. sojina* affected seed germination. Incidence (%) values were transformed to $\sqrt{\chi + 1}$ to reduce skewness and stabilize distributional variance. Levene and Shapiro-Wilk tests confirmed the homogeneity and normality of the transformed data, respectively. The analyses were performed with the car (Fox and Weisberg 2011) and stats packages of R (R Core Team 2017), respectively. The data were subjected to analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

Results

Incidence of *C. sojina* in the samples

The pathogen was detected in 26/78 samples. No other fungi than *C. sojina* were present. Incidence of the pathogen in the samples ranged from 0.5 to 30.87% (overall mean of 1.9%) based on the water restriction blotter assay. Fasciculate conidiophores and conidia of *C. sojina* developed abundantly on the seed surface, mainly in the hilar region. The conidia were hyaline, 2–8 septate and 35.1–77.2 μm (56.7 ± 11.9) × 7.0–7.7 μm (7.4 ± 0.3) in size.

Effect of media on the detection

The mean incidences ranged from 0.62 to 3.72% among the eight methods tested for sample 1, from 2.24 to 5.16% and from 21.95 to 37.21% among the eleven methods tested for sample 2 and sample 3 respectively (Table 1). There were no statistically significant differences between culture media to detect *C. sojina*. Only for sample 1 the vegetable broth light agar had a lower

Table 1 Mean incidence (%) of *Cercospora sojina* in soybean seeds in different media for fungi detection

Media	Sample 1[a]	Sample 2[b]	Sample 3
Carrot soft agar	1.42 ab	4.96[ns]	21.95[ns]
Carrot agar	2.54 ab	4.19	36.27
Tomato sauce agar	1.51ab	4.26	33.71
Soybean leaves agar	2.45 ab	4.36	26.75
Vegetable broth agar	1.67 ab	4.78	30.78
Vegetable broth light agar	0.62 b	4.47	32.45
Vegetable broth light (no salt)	3.72 a	3.90	37.21
Water restriction blotter test	2.78 ab	4.19	28.48
V8 juice agar	–	5.16	30.28
Potato dextrose agar	–	3.45	33.41
Synthethic nutrient agar	–	2.24	23.94
CV	26.71%	22.27%	10.73%

[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$

[b] ns = not significantly different at $P < 0.05$

percentage of infection with respect to the vegetable broth light (no salt). The pathogen did not affect germination. Fasciculate conidiophores and conidia of *C. sojina* developed abundantly on the seed teguments and characteristics did not differ among the methods.

Effect of fungicide seed treatments

In experiment 1, pyraclostrobin + thiophanate-methyl and carbendazim + thiram eradicated the pathogen in seed at all infection level tested ($P < 0.05$) (Fig. 1). Fludioxonil + metalaxyl also eradicated the pathogen but only when applied on soybean seed samples with low incidences of *C. sojina*. On samples with higher incidences (around 10%), fludioxonil + metalaxyl was less effective.

In experiment 2, only fludioxonil + metalaxyl + carbendazim, thiram + carbendazim and thiophanate-methyl + pyraclostrobin eradicated the pathogen in seed at all infection level tested ($P < 0.05$) (Fig. 2). Trifloxystrobin + metalaxyl, carboxin + thiram and thiram also eradicated the pathogen but only when applied on soybean seed samples with low incidences of *C. sojina*. Fludioxonil + metalaxyl and ipconazole + metalaxyl were not effective (without differences with the control at all infection level tested).

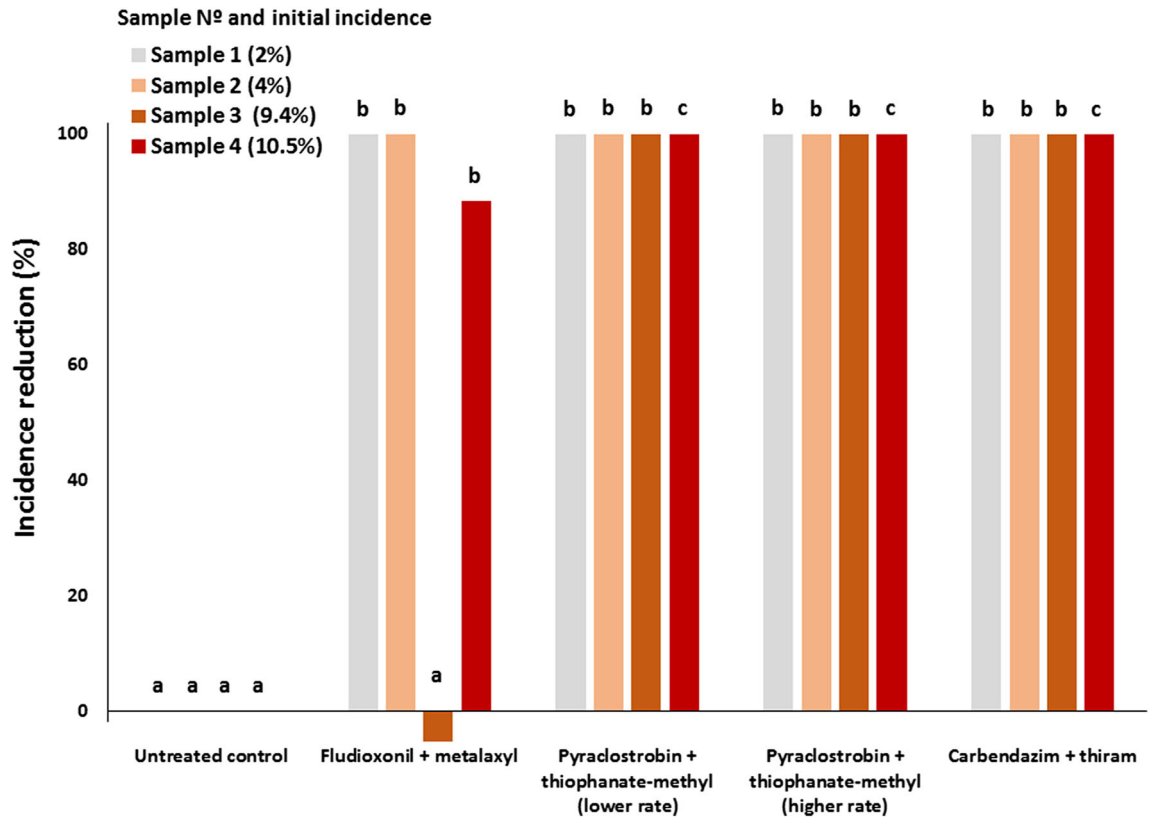

Fig. 1 Effect of four fungicide treatments on reduction of *Cercospora sojina* incidence in four field seed samples with varying levels of incidence, as determined by the water restriction blotter method (Experiment 1). Different letters on top of bars indicate significant differences ($P < 0.05$) among treatments within the same sample

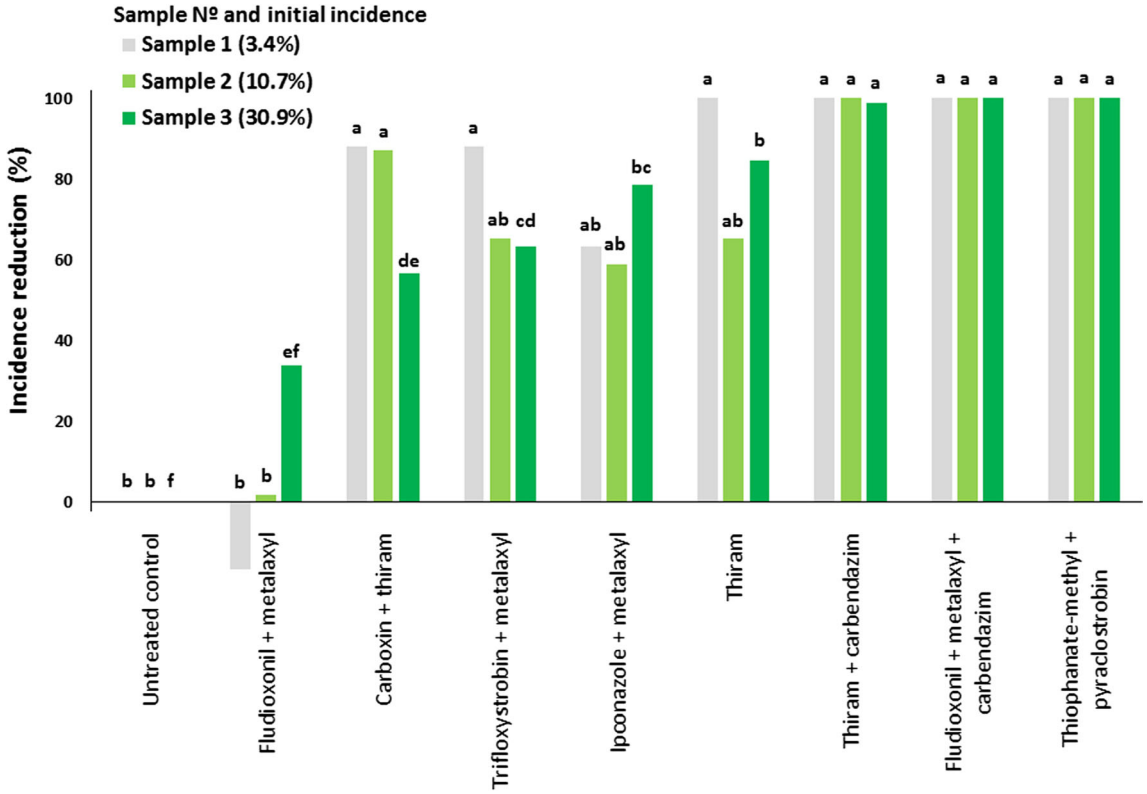

Fig. 2 Effect of eight fungicide treatments on reduction of *Cercospora sojina* incidence in three field seed samples with varying levels of incidence, as determined by the water restriction blotter method (Experiment 2). Different letters on top of bars indicate significant differences ($P < 0.05$) among treatments within the same sample

In experiment 3, only fludioxonil + metalaxyl + thiabendazole, fludioxonil + metalaxyl + thiabendazole + thiamethoxam and thiram + carbendazim eradicated the pathogen in seed at all infection level tested ($P < 0.05$) (Fig. 3). Fludioxonil + metalaxyl and trifloxystrobin + metalaxyl were not effective (without differences with the control at all infection level tested), except for trifloxystrobin + metalaxyl in sample 2, which had intermediate control levels. In all experiments, no toxicity on seed germination due to fungicide was observed at the evaluated doses.

Discussion

Cercospora sojina-infected soybean seed was previously associated with the dispersal of FLS pathogen (Lehman 1934) and is likely the means of introduction and spread of FLS in the agricultural pampas region of Argentina (Carmona et al. 2009). Detection of soybean pathogens on seed is commonly conducted using the routine standard blotter test or the standard agar test without suppression of germination, and with or without surface sterilization of the seeds before plating (Yorinori 1989; Machado et al. 2002; Mathur and Kongsdal 2003). In order to facilitate the routine analysis, 2,4-D and water restriction can be applied in blotter tests to suppress germination (Machado et al. 2002). When using the blotter test for *C. sojina* infections, water restriction was as effective as 2,4-D to suppress germination and produced similar results. In fact, all methods were equivalent, even though the vegetal broth light no salt agar was the most sensitive method for sample 1. However, water restriction blotter method is a more time and cost-effective method, besides facilitating and speeding up identification of *C. sojina*. Most of the methods recommended for seed health testing both using the agar-based and blotter tests have not been tested in relation to accuracy and reproducibility (Yorinori 1989; Machado et al. 2002; Mathur and Kongsdal 2003). Although water restriction method had been subjected to a pilot inter-laboratory study (Scandiani et al. 2009c) further comparisons using more heterogeneous samples are required to confirm these results.

In the seed treatment experiments, benzimidazole fungicides were more effective to inhibit sporulation of *C. sojina* regardless the infection levels of the seeds. The ability to eradicate the fungus makes suitable for

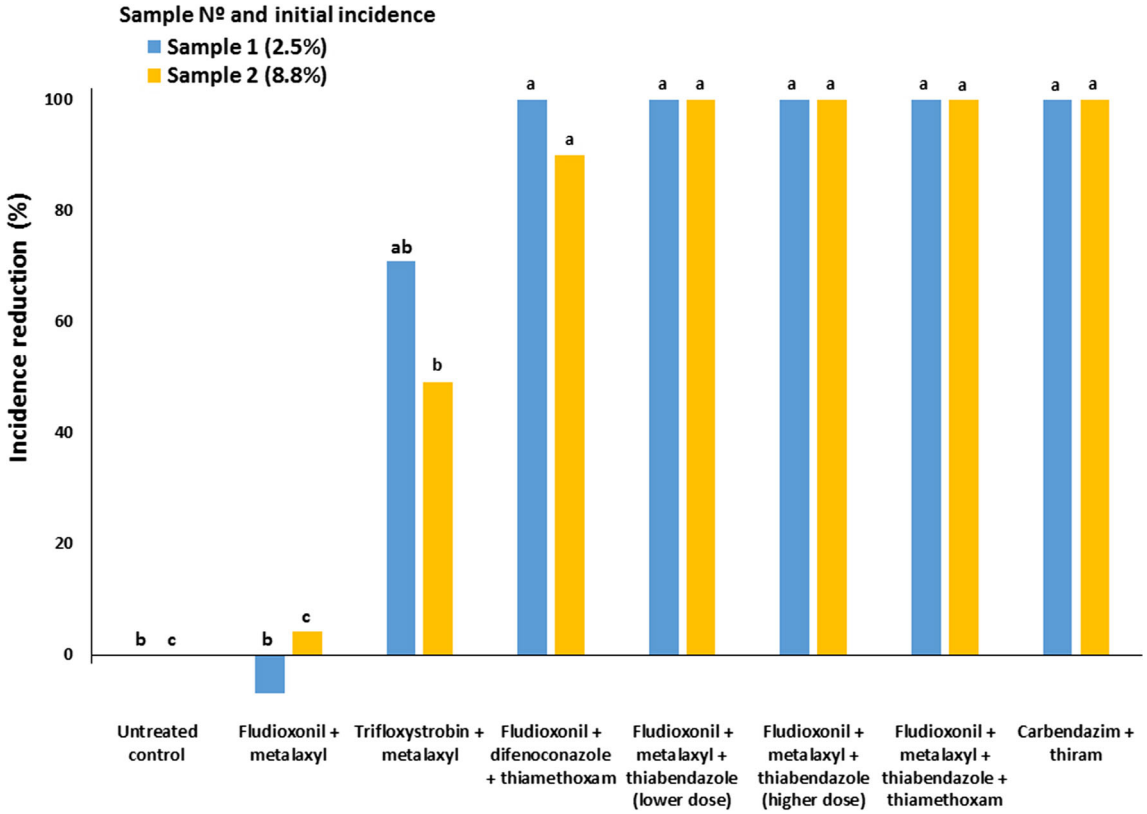

Fig. 3 Effect of seven fungicide treatments on reduction of *Cercospora sojina* incidence in two field seed samples with varying levels of incidence, as determined by the water restriction blotter method (Experiment 3). Different letters on top of bars indicate significant differences ($P < 0.05$) among treatments within the same sample

treating infected seeds prior to planting. The efficacy of benzimidazoles against *Cercospora spp.* has been reported in previous studies (Mwase and Kapooria 2000). Similarly, for other seedborne pathogens such as *Ascochyta rabiei*, thiabendazole (from the family of benzimidazoles) allowed to eradicate the fungi from the seed (Reddy and Singh 1984). Due to the potential development of resistance to benzimidazoles (Davidse and Ishii 1995), it is advisable to apply benzimidazole in mixture with fungicide from other group (mode of action). Although *C. sojina* has been detected on soybean seed (Lehman 1934; Yorinori 1989), there is limited literature data on the topic. For instance, Yorinori (1989) reported that seed treatment with the fungicides thiram plus thiabendazole has been effective in reducing seed transmission.

In conclusion, this study confirms *C. sojina* as an important soybean seed-borne pathogen in Argentina and suggests the water restriction blotter test as recommended for detecting the fungus in the seed. Seed treatments with a full effective fungicide in addition to crop rotation may help to suppress the disease and minimize the risk of seed infection. For FLS-free fields, information on seed health quality is essential for decision-making on seed treatment to further prevent the introduction of the various races of the pathogen into new areas. Further research should focus on quantifying: i) relationship between seed infection and FLS epidemics (and yield losses) in the field, ii) seed-to-plant transmission rate, iii) thresholds of fungal incidence in the seeds for chemical treatment and iv) sensitivity levels to commonly used fungicides.

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