International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 23, No. 6, p. 1-9, 2023

RESEARCH PAPER OPEN ACCESS

Morphological diversity, pathogenicity and biofungicides efficacity on *Cercospora arachidicola* strains causal agent of early leaf spot disease of groundnut

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Article published on December 10, 2023

Key words: Groundnut, *Cercospora arachidicola,* Diversity, Pathogenicity, Biofungicide

Abstract

Fungal diseases are one of major constraints on groundnut production in Burkina Faso. Among these diseases, early leaf spot caused by *Cercospora arachidicola (*Hori.) is one of the most important economic diseases of groundnut. Aim of contributing to search effective control methods against this disease, we undertook the present study, which consisted in (i) study morphological diversity of different *Cercospora arachidicola* isolates (ii) study the level of pathogenicity of *Cercospora arachidicola* strains identified (iii) evaluate the efficacy of some bio-fungicides on the strains identified. The study was carried out in 14 villages in the Hauts Bassins and Boucle du Mouhoun regions of Burkina Faso with regard to prospecting and sample collection, and at the INERA Bobo Dioulasso plant pathology laboratory for isolation, identification, pathogenicity and biofungicide efficacy testing. A total of five strains of *Cercospora arachidicola* were identified. The pathogenicity test was used to classify the five strains according to their virulence. In decreasing order of virulence, the strains were Fara, Darsa, Logo, Santi and Kod. In vitro evaluation of biofungicide efficacy shows that PLANSAIN biofungicide provides better control of *Cercospora arachidicola* strains. *Trichoderma hazanium,* the active ingredient in PLANSAIN, inhibits the radial growth of *Cercospora arachidicola* strains to a greater extent.

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Introduction

Groundnut, whose scientific name is *Arachis hypogeae* L.*, was* first described in 1753 by Linnaeus (Gillier and Sylvestre, 1969), is a crop that adapts too many climatic zones. It is a nutrient-rich herbaceous legume. The seeds contain 22 to 32% protein, 34 to 54% fat and around 12% carbohydrates (Nyabyenda, 2005). Groundnuts are a fully edible, multi-purpose plant. It is highly effective in the treatment of tooth decay (Ntare, 2007), is widely used in the food industry (Hubert, 2000) and in the manufacture of cattle meal (Subba Rao, 1987). The groundnut plant is used for soil fertilization and cultural ceremonies by Bissa and Gourounsi ethnic groups in Burkina- Faso (Bantenga, 2010). In 2019, groundnut production stood at nearly 48.7 million tonnes, with 29.6 million ha under cultivation, 34.2% of which comes from Africa (Faostat, 2020). As such, it occupies a prime position in crop production. Burkina-Faso, 6ème groundnut-producing country in West Africa, records an average production of 354714.4 tonnes on a surface area of 447529.9 ha, i.e. 794.63 kg/ha (Faostat, 2020). This crop is one of Burkina Faso's main cash crops after cotton. Despite its importance, groundnut production in Burkina Faso faces a number of problems. These include the absence of a sound policy to promote the crop (Sofivar, 1998), poor spatio-temporal rainfall distribution (Gillier and Sylvestre, 1969), and insect pests and diseases that attack the crop. Among the many constraints on groundnut production, biotic factors, particularly leaf diseases, are a real handicap (Savary *et al.*, 1987).

Losses caused by early and late leaves spot diseases are enormous, and can reach 80% of production in cases of severe attack (Gillier and Sylvestre, 1969). Early and late leaves spot diseases can be recognized by the brown and black lesions on the upper and lower surfaces of the leaves. Early leaf spot disease is the most important disease in sub-Saharan Africa, particularly in Burkina Faso. To combat this pathology, which has a major impact on groundnut yields; several control methods have been developed. These include adapted cultural practices, chemical control, the use of resistant varieties, the use of biopesticides and an integrated approach to the disease. However, in view of climate change we are facing, it is more than necessary to find an alternative that both controls early leaf spot disease and respects environment and human health.

Several studies have already been carried out to develop biopesticides against fungal diseases. Sharma and Sain in 2003 showed that *Trichoderma harzianum*, *Trichoderma viride* and *Pseudomonas fluorescence* control *Aspergillus flavus* in groundnuts. Koïta *et al.* (2010) and Koïta *et al.* (2012) have demonstrated the efficacy of aqueous plant extracts in controlling leaf spot diseases of groundnut. Krishna *et al.* 2005 showed that an acellular bacterial filtrate of the genus *Pseudomonas sp* controls the germination of *Cercospora* and rust spores.

The aim of this study was to identify different strains of *Cercospora arachidicola*, causal agent of early leaf spot disease, in order to better channel efforts to combat this disease, while protecting people and the environment by limiting chemical products use. In generally this study was to identify the different strains of *Cercospora arachidicola*, causal agent of early leaf spot disease. Specifically, the aims were to: (i) study the morphological diversity of the different isolates of the fungus (ii) assess the virulence levels of the strains identified (iii) evaluate the efficacy of bio-fungicides on the strains identified.

Materials and methods

Study sites

The study was carried out in the laboratory of the Institut de l'Environnement et de Recherche Agricole (INERA) located in Farako-bâ near Bobo Dioulasso. The survey and sample collection took place in the Hauts Bassins and Boucle du Mouhoun regions. In all, groundnut farms were surveyed in 14 villages, including 13 in Hauts Bassins region and one (01) in Boucle du Mouhoun region.

Plants material

The plant material consisted of thirty-one (31) groundnut leaf samples from the Boucle du Mouhoun and Hauts Bassin regions showing symptoms of early leaf spot disease of groundnuts (Table 1). These samples were used to isolate the strains of *Cercospora arachidicola*.

Table 1. Origin of samples used for isolation of *Cercospora arachidicola* strains

Samples	Villages	Regions
S01	Matourkou	Hauts Bassins
S02	Samagan	Hauts Bassins
S03	Samagan	Hauts Bassins
S04	Farako-Bâ	Hauts Bassins
S05	Farako-Bâ	Hauts Bassins
S06	Dar Salami	Hauts Bassins
S07	Dar Salami	Hauts Bassins
S08	Dar Salami	Hauts Bassins
S09	Dar Salami	Hauts Bassins
S10	Dar Salami	Hauts Bassins
S11	Lafiabougou	Hauts Bassins
S12	Lafiabougou	Hauts Bassins
S13	Logofourousso	Hauts Bassins
S14	Logofourousso	Hauts Bassins
S15	Koumi	Hauts Bassins
S16	Koumi	Hauts Bassins
S17	Dafinso	Hauts Bassins
S18	Dafinso	Hauts Bassins
S19	Dafinso	Hauts Bassins
S20	Santidougou	Boucle du Mouhoun
S21	Santidougou	Boucle du Mouhoun
S22	Bolibana	Hauts Bassins
S23	Bolibana	Hauts Bassins
S24	Banankélédaga	Hauts Bassins
S25	Banankélédaga	Hauts Bassins
S26	Koro	Hauts Bassins
S27	Koro	Hauts Bassins
S28	Borodougou	Hauts Bassins
S29	Borodougou	Hauts Bassins
S30	Niamadougou	Hauts Bassins
S31	Niamadougou	Hauts Bassins

The TS32-1 variety was used for pathogenicity test of the various strains identified and biopesticides efficacy test against *Cercospora arachidicola* strains identified. This Spanish-type variety is a cross between Spantex and TE 3, with a 90-day cycle and a high germination rate of up to 98%. TS32-1 is a non-dormant variety and is susceptible to early leaf spot disease. It was breed by INERA and popularized in Burkina-Faso. It was used for pathogenicity and biopesticide efficacy tests on different strains of the fungus.

Bio-fungicides used

Two biofungicides and one synthetic fungicide were used to test their efficacy against the different strains of *Cercospora arachidicola* identified. These were

Plantsain: 4% Gamma Lactone from *Trichoderma* extracts, 1% Citrus terpene oil, 0.5% Clove oil, 2% Magasium oxide, 0.1% Maganese, 0.1% Zinc. Fertisain: 3% Gamma Lactone from *Trichoderma* extracts, 1% Citrus Terpene Oil, 0.5% Clove Oil, 2% Magasium Oxide, 0.1% Maganese, 0.1% Zinc.

Azox: It is a broad-spectrum synthetic systemic fungicide containing azoxystrobin at a dose of 250g/l in a concentrated suspension as active ingredient.

These biofongicides were provides by Bioprotect Foundation.

Samples collection

Symptom identification and sample collection involved groundnut plants at vegetative growth stages. Samples were collected randomly along field diagonals. Plants whose leaves showed symptoms characteristic of early leaf spot disease were collected. Once collected, the samples were placed in envelopes bearing the date, location and geographical coordinates. The samples were then sent to the laboratory for incubation on blotting paper.

Samples incubation

Incubation began with sterilization of the equipment. Once the equipment had been sterilized, we moved on to sample preparation, which involved placing the blotting papers in petri dishes. Next, the symptomatic leaves were rinsed twice with distilled water before being placed in petri dishes. Finally, the petri dishes were labelled and placed in an incubation room for 72 hours at a temperature of 25°C, in the presence of UV light and alternating light and darkness.

Preparation of groundnut culture medium

Cercospora arachidicola being an obligate fungus, we prepared a specific groundnut-based medium for its isolation. To this end, 32 g of groundnut flour and 20 g of Agar were mixed in 1000 ml of distilled water in a Pyrex jar. After homogenizing the mixture, it was placed in an autoclave for sterilization at 120°C for 30 minutes.

Isolation and strain purification

To obtain the isolates, an antibiotic (streptomycin sulfate) was added at a dose of 0.25 g per 1000 ml to the prepared culture medium. This medium was distributed in Petri dishes at a rate of 25 ml per dish. From the fruiting bodies of the fungus obtained on organs incubated in a humid chamber, a cluster of mycelia and conidia were removed with a needle and deposited in a Petri dish containing the culture medium. The resulting Petri dishes were incubated under 12 h of near-UV light alternating with 12 h of darkness for five 5 days. A purification operation was performed to obtain pure colonies. Microscopic observations were made to confirm the identity of each colony.

Strain characterization

The aim of this study was to identify the morphological diversity that might exist between strains. Two characteristics based on visual observation and measurements were selected for morphological characterization of the samples. These are:

Mushroom coloration: visual observation was used to identify the different colors of stump mycelium.

Radial growth: this consisted in evaluating changes in mycelial length along the perpendicular axes of the petri dishes.

In vitro pathogenicity test

The in vitro pathogenicity test enabled us to determine the virulence level of 05 strains of *Cercospora arachidicola* identified on TS32-1. These were Darsa from Dar Salami, Fara from Farako-bâ, Kod from Kodougou, Logo from Logofourouso and Santi from Santidougou.

An Agar solution composed of 10 g of Agar in 500 ml of distilled water was used as a carrier in the petri dishes at a rate of 25ml/plate. Leaves were collected randomly from healthy young leaves 10 and 20 days after sowing.

Two experiments were carried out with leaves aged 10 and 20 days after sowing, respectively, using a total randomization design with three (03) replicates. A total of 6 treatments were studied:

T0: No inoculation (absolute control)
T1: Inoculation with Darsa strain;
T2: Inoculation with Fara strain;
T3: Inoculation with Santi strain;
T4: Inoculation with Logo strain;
T5: Inoculation with Kod strain.

In each petri dish containing the agar support, 4 groundnut leaflets (10 or 20 days old, depending on the experiment) were placed on the underside. Inoculation was carried out using a 0.5 cm diameter tube, which was used to collect a mycelial fragment from the strains. These mycelial fragments were then placed on the upper surface of each of the 04 leaflets.

Observations concerned symptomatic manifestation of early leaf spot on infested leaves. Scoring was done every 48 hours using the 9-point scoring scale of Subrahmanyam *et al.* (1995).

Biofungicides efficacity test on Cercospora arachidicola strains identified

Fungicide efficacy test consisted in evaluating the efficacy of two bio-fungicides on the strains of *Cercospora arachidicola* identified. The five strains identified were isolated in petri dishes. A split-plot experiment with two (02) replicates was conducted in the laboratory to assess the efficacy of these bio-fungicides. The factors studied were the efficacy of the different bio-fungicides (primary factor) and the dose of bio-fungicides (secondary factor). The levels of the main factor (bio-fungicide efficacy) are as follows:

P0: No fungicide treatment (absolute control);
P1: Treatment with PLANTSAIN bio-fungicide;
P2: Treatment with FERTISAIN bio-fungicide;

P3: Treatment with AZOX synthetic fungicide.

The levels of the secondary factor (treatment dose) are:

D1: Treatment with recommended dose of 75ml/16l for PLANTSAIN, 50ml/16l for FERTISAIN and 35ml/10l for AZOX;

D2: Treatment with experimental dose, which represents 5/4 of normal dose, i.e. 93.75ml/16l for PLANTSAIN, 62.5ml/16l for FERTISAIN and 43.75ml/10l for AZOX.

Treatments were carried out in petri dishes containing the strains from the corresponding localities and in accordance with the doses. Using a tube, 0.8 cm diameter colony explants of the strains were deposited in petri dishes containing the treated groundnut culture media. Product efficacy was assessed by measuring mycelial growth along the perpendicular axes of petri dishes. Measurements were taken every 02 days. This was done to assess the inhibitory capacity of fungicides used on evolution of different strains of *Cercospora arachidicola*.

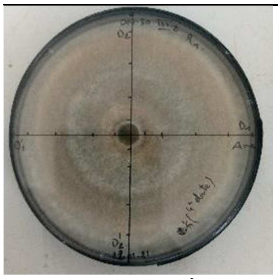
Data analysis

The data obtained were entered into an Excel spreadsheet and an analysis of variance was performed using XLStat Pro 2020 software. Means were compared using Duncan's test at 5% threshold. Isolate images were viewed with a magnifying glass to morphologically differentiate colonies from different isolates.

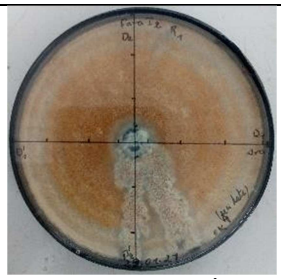
Results

Morphological diversity of Cercospora arachidicola strains

Color	Greyish center with white edges	Color	Dark pink center with white borders
Mycelium size	Dense	Mycelium size	Dense
Borders	Precise contour	Borders	Precise contours



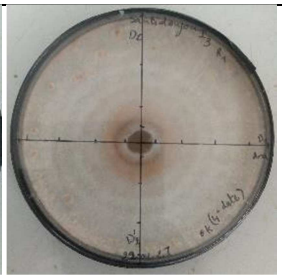
Darsa strain



Fara strain

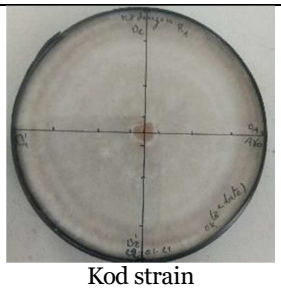
Color	White with pink flecks	Color	Light pink to white
Mycelium size	Dense	Mycelium size	Dense
Borders	More or less defined	Borders	Very precise

Logo strain



Santi Strain

Color	Blanche
Mycelium size	Very dense
Borders	Precise



Kod strain

Fig. 1. Morphological diversity of *Cercospora arachidicola* strains

The physical characteristics of different strains of *Cercospora arachidicola* identified through visual observations are recorded in Fig. 1. A total of five (05) strains were described on the basis of visual observation of colonies. Based on frequency, these strains were distributed by survey locality. We did not observe more than one strain in the same locality. The characteristics of the strains identified are as follows:

Darsa strain

Derived from samples collected at Dar Salami locality. It is characterized by abundant mycelium. The colony is grayish in the center with well-defined white borders.

Fara strain

Isolated from samples collected at Farakô-ba, near INERA station in Bobo Dioulasso. The colony is pink and white with an alternating pattern. The strain's mycelium is fairly dense, with sharp edges.

Logo strain

From Logofourousso isolates. This strain shows a whitish colony with pink spots. It is also characterized by abundant mycelium with more or less regular outlines.

Santi strain

Derived from Santidougou isolates located on the Bobo-Dédougou axis. Santi strain can be identified by a colony with a gradient of pink to white coloration from the center to the edges. The mycelium is abundant and regularly outlined.

Kod strain

Derived from isolates from Kodougou in the Boucle du Mouhoun region. In contrast to the other strains, the Kod strain shows a mycelium with regular contours, very abundant and white in color.

Pathogenicity test of Cercospora arachidicola strains

Table 2 shows the early leaf spot disease average severity scores for the different strains of *Cercospora arachidicola* tested for virulence on 10- and 20-day-old leaves of the TS32-1 groundnut variety in vitro. Severity scores ranged from 1 obtained with the control treatment (T0) to 7 obtained with Darsa and Santi strains and from 1 (T0) to 6 obtained with the Logo strain respectively on leaves of 10 and 20 days after sowing. The Darsa strain was the most virulent, with an average score of 7 on 10-day-old leaves, following by Logo and Darsa strains on 20-day-old leaves, with severity scores of 6 and 5 respectively. The Kod strain was the least virulent, with a severity

score of 4 and 2 respectively on leaves aged 10 and 20 days after sowing. Overall, the analysis of variance showed a significant difference between strains for leaves 10 days old.

Efficacy test of bio-fungicides on Cercospora arachidicola strains

Table 3 shows the results of analysis of variance and means comparisons for the last measurement of mean diameter of radial growth of *Cercospora arachidicola* strains.

Darsa strain

The radial growth diameters of the various treatments on the Darsa strain ranged from 1.45 to 5.975 cm, obtained respectively with PLANTSAIN and the control treatment. PLANTSAIN, which proved the most effective against this strain, was followed by AZOX (reference control). Overall, the analysis of variance showed a highly significant difference between the fungicides tested. However, no differences were revealed for the doses tested.

Table 2. Average severity scores of *Cercospora arachidicola* strains on groundnut leaves 10 and 20 days after sowing

10-day-old leaves DAS		20-day-old leaves DAS	
Straim	Score	Straim	Score
Darsa	7 b	Logo	6 c
Santi	7 b	Darsa	5 bc
Logo	5 b	Santi	4 bc
Fara	5 b	Fara	4 bc
Kod	5 b	Kod	2 ab
Control (check)	1 a	Control (check)	1a
Average	5.06	Average	3.83
Standard deviation	2.60	Standard deviation	2.15
F Proba	0.03*	F Proba	0.09

Table 3. Variation analysis of bio-fungicide efficacy on *Cercospora arachidicola* strains

Fungicides	Strains				
	Darsa	Fara	Santi	Kod	Logo
Azox	2,725 b	3,6625 a	3.4625 bc	3.9375 bc	3.1875 ab
Fertisain	5,7 a	5,7625 a	5.525 ab	6.25 ab	5,6875 a
Plansain	1,45 b	1,6625 b	1,75 c	1,875 c	1,8375 b
To	5,975 a	6,075 a	6,125 a	6,775 a	6,275 a
Fungicide (F)	0,008**	0,0356*	0,0214*	0,0077**	0,0214*
Doses (D)	0.2904ns	0.1364ns	0.5582ns	0.2995ns	0.5582ns
F*D	0.2802ns	0.0763ns	0.679ns	0.1051ns	0.679ns
CV	14,51	8,93	21,29	12,26	18,92

Fara strain

The radial growth diameters of the different treatments on Fara strain ranged from 1.6625 (PLANTSAIN) to 6.075 (untreated control). As in the case of the Darsa strain, PLANTSAIN proved the most effective against this strain. This was followed by the AZOX (reference control), which recorded a radial growth diameter of 3.6625 cm. Analysis of variance showed a highly significant difference between the fungicides tested.

Santi strain

Radial growth diameters of treatments on Santi strain ranged from 1.75 to 6.125 obtained with PLANTSAIN and the control treatment. As in the case of the Darsa and Fara strains, PLANTSAIN proved the most effective against Santi strain. Analysis of variance showed a significant difference only between the fungicides tested.

Kod strain

For Kod strain, the smallest radial growth diameter (1.875 cm) was recorded by PLANTSAIN and the largest diameter (6.775 cm) by the control treatment. As in the case of the three previous strains (Darsa, Fara and Santi), PLANTSAIN proved the most effective against this strain. Analysis of variance also showed a significant difference only between the fungicides tested.

Logo strain

The smallest radial growth diameter was recorded by PLANTSAIN at 1.8375 cm, and the largest diameter by the untreated control at 6.275 cm. Thus, PLANTSAIN proved effective against the Logo strain. Analysis of variance showed a significant difference only between the fungicides tested.

Overall, coefficients of variation (CV%) for all strains ranged from 8.93 (Fara) to 21.29 (Santi). These points to relatively low variability between strains.

Discussion

Our research results revealed the existence of morphological diversity between strains of *Cercospora arachidicola*. This diversity could be due to the influence of the environment, given that the isolates were from different origins. In fact, we observed no diversity between isolates from the same locality. Furthermore, the pathogenicity test enabled us to perceive a difference in behavior depending on the strains isolated, which corroborates the hypothesis that there is variation between strains of *Cercospora arachidicola* depending on the agro-ecological zone. Our results are in accordance with those of Minougou (2006), who showed that rainfall is a determining factor in the development of early leaf spot disease of groundnut.

Analysis of variance for pathogenicity test showed a significant difference between strains for early leaf spot disease severity scores. This indicates the existence of diversity in the impact of the disease, depending on the strain. This could explain the different levels of virulence and aggressiveness of the disease in different regions. The results of pathogenicity test also showed a high virulence of strains on leaves 10 days old after sowing compared with leaves 20 days old after sowing. This suggests that leaf age influences the plant's level of resistance to the pathogen responsible for early leaf spot disease. This observation was made by Gillier and Sylvestre in 1969, who demonstrated that the rate of spread of *Cercospora arachidicola* slows down when leaves are thicker. So, beyond the morphological diversity perceived in the identification phase, there is a variation in the level of virulence between strains. The Logo, Darsa and Santi strains were the most virulent, followed by Fara and Kod strains.

Efficacy test of bio-fungicides on strains identified PLANSAIN bio-fungicide as the most effective inhibitor of radial growth of *Cercospora arachidicola* strains. We can therefore conclude that *Trichoderma harzianum,* the active ingredient in this bio-fungicide, has an effect on the control of *Cercospora arachidicola*. Furthermore, our results are in accordance with the studies of several authors indicating that *Trichoderma harzianum* isolates have an inhibitory effect on certain groundnut mycoses, notably *Aspergillus flavus* and telluric fungi attacking the roots (Biswas and Sen, 2000; Kishore *et al*., 2001; Rakholiya *et al*., 2010; Bagwan, 2011; Sreedevi *et al*., 2011; Sreedevi *et al*., 2012). Thus, in the light of our study, we can confirm that in addition to controlling *Aspergillus flavus*, *Trichoderma harzianum* has an inhibitory effect on the growth of *Cercospora arachidicola*. In addition to early leaf spot disease, PLANSAIN could be used to control other fungal diseases on various crops. *Trichoderma harzianum,* the active ingredient of this biofoncgicide, has a broad-spectrum antifungal effect. Indeed, Sharma *et al*, (2014) demonstrated the efficacy of *Trichoderma harzianum-based* biofoncigides on several fungal diseases of various crops.

The FERTISAIN applied showed little control over the five strains, a result justified by the fact that this product is basically designed for bio-control of telluric fungi. It is proposed as a root fertilizer to control mainly telluric fungi.

Conclusion

In undertaking this study of diversity of *Cercospora arachidicola,* strains, causal agent of early leaf spot disease of groundnuts, we set ourselves the objective to identify potential strains of this mycosis and, at the same time, assessing their level of virulence. A study of morphological diversity revealed the existence of five (05) strains of *Cercospora arachidicola* based on their coloration, four of which are found in the Hauts Bassins region and one (01) in the Boucle du Mouhoun region.

Pathogenicity test showed that the strains have a wide range of virulence, which may explain why the disease is not controlled from one region to another. The Darsa, Logo and Santi strains showed a very high level of virulence, while the Kod strain was less severe. This test is very important for researchers in that, depending on the level of attack on the plant, the strain responsible can be detected and treatments against this fungus can be more effective.

Biofungicides efficacity test on strains of *Cercospora arachidicola* has identified PLANSAIN as an effective fungicide against this disease. It will be better suited to the control of fungal leaf diseases of groundnuts.

The results obtained through our work constitute significant advances for research. However, a morphological identification of the strains does not allow us to perceive the extent of the existing diversity between the strains of *Cercospora arachidicola*. In future, therefore it would be advisable to assess molecular diversity and extend the study area to cover the whole country. This will provide more information on strains that could help improve the formulation and dosage of fungicides with *Trichoderma harzanium* extracts for more efficient control. In addition, if the tests in controlled environments have proved conclusive, the extension of the studies to real environments will enable other factors to be considered.

Acknowledgments

The authors thank warmly the whole team of INERA, University Joseph Ki-Zerbo and University of Dedougou for their active participation and financial support to this study. We thank also Bioprotect Foundation for their collaboration and providing the biofuncigides.

Disclosure of conflict of interest

The authors declare no conflicts of interest regarding the publication of this paper.

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