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Isolation and *in-vitro* assessment of antagonistic activity of *Trichoderma* spp. against *Magnaporthe oryzae* Longorola strain causing rice blast disease in Mali

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***Pyricularia oryzea (Magnaporthe oryzae)* causes blast diseases in rice (*Oryza sativa*) in Mali. The losses could reach 90% of production during rainy weather conditions. Isolation and characterization of *M. oryzae* and *Trichoderma* species were carried out to assess the importance and distribution of the pathogen and antagonist *Trichoderma* species in rice fields in Sikasso (Mali), and select, *in vitro, Trichoderma* species with high pathogen biocontrol activity. In the pathogen isolation, only one isolate of *M. oryzae* were obtained, while 12 *Trichoderma* isolates were obtained. In the fungal growth tests three isolates of *Trichoderma*: *Trichoderma harzianum* S31, *T. harzianum* S32, and *T. harzianum* S33 highly inhibited the growth of the pathogen with a coefficient of antagonism of 0.55, 0.71 and 0.78 respectively. These isolates were selected for further greenhouse and field tests.**

Keys words: Rice blast disease, *Trichoderma*, *Magnaporthe oryzae*, *Pyricularia oryzea*, antagonism, *Oryza sativa*, Mali.

INTRODUCTION

Rice (*Oryzae sativa*) is a staple food and cereal crop for more than half of the population in Mali where agriculture drives the national economy (ZEF, FARA, IER (2017)) Mali is one of the top rice producers in West Africa with a 3.19 million tons of rice produce in 2019 (FAOSTAT, 2019). Unfortunately, the country is also particularly vulnerable to agricultural diseases (Gurr et al., 2011), mainly rice blast diseases, which limit rice yields to below the global average, threatening smallholder farmers' livelihoods as well as food and economic security (USDA, 2012; Asibi et al., 2019; Soullier et al., 2020).

The fungal plant pathogen *Magnaporthe oryzae*, involved in causing serious blast diseases in rice in Mali, is very difficult to manage. At present, the losses caused

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by this pathogen could reach 30 to 90% of the rice production in the affected areas (Savary et al., 2000; Khemmuk, 2016). For that, chemicals, compounds are widely used to control blast disease pathogens (Dougoud et al., 2018). *Pyricularia oryzae (M. oryzea)* is the causal agent of this disease on wheat (Tembo et al., 2020), maize (Pordel et al., 2021) and *Panicum repens* (Well et al., 2005). It can cause blast disease on the three hosts. Pordel et al. (2021) in pathogenicity assays in greenhouse revealed that strains from maize can infect barnyard grass and conversely.

Biological control is a promising tool to maintain good level of agricultural production while reducing the release of polluting chemical pesticides to the environment. Many researches showed that soil microorganisms, mainly fungi species, are promising as biocontrol agents (Swain et al., 2018; Sood et al., 2020; Es-Soufi et al., 2020). Out of these microorganisms, *Trichoderma* species, filamentous fungi previously considered to be culture contaminants species, are common inhabitant of rhizosphere and contribute to control of many soil-borne plant-diseases caused by fungi (Chuaki et al., 2002; Bastakoti et al., 2017). *Trichoderma* spp., have been largely studied as biological control agents against plant pathogens (Rivera-Méndez et al., 2020; Alfiky and Weisskopf, 2021; Bastakoti et al., 2017). In recent years, considerable success has been achieved in the control of plant diseases by the use of *Trichoderma* isolates which have become commercially available as biocontrol agents (Woo et al., 2014; Es-Soufi et al., 2020). Chou et al. (2019) combined the use of *Trichoderma harzianum* and a resistant rice variety, considered as sustainable approaches to reduce yield losses and to cope with recent restrictions on fungicide use to manage blast disease, showed that *T. harzianum* reduced the incidence of leaf blast and neck blast on IR504 (susceptible strain), but its efficacy was not consistent and the magnitude of disease suppression by T. harzianum was higher for neck blast than for leaf blast. Also, Mouria et al. (2018) applying *T. harzianum* at a concentration of 10^8 spores/ml, in alternation with the mancozeb at 1000 ppm against rice blast and rice leaf spot and the pyrazophos at 750 ppm against blast; showed that the alternation of pyrazophos and *T. harzianum* reduced blast at a rate similar to that noted when pyrazophos is used alone (that is, respectively 90.5 and 89.1%). This percentage is better than that recorded following treatment by *T. harzianum* alone (78.4%). Mancozeb alternated with *T. harzianum* reduced blast at a rate of (83.49%) compared with the fungicide or the antagonist alone (77 and 78.4%). Nevertheless, essential knowledge concerning the distribution and efficacy of *Trichoderma* species as antagonists for their effective use to act against rice blast pathogens is lacking in Mali. That is why, the present research was undertaken to explore the possibility of isolating and selecting *Trichoderma* strains with high biocontrol activity against rice blast disease pathogen.

MATERIALS AND METHODS

Source of isolates and pathogen isolation

Samples of contaminated soil (5) from rice fields (lowland), leaves (15) and panicles (3) of diseased rice were collected at the Longorola research station of the ″Institut d'Economie Rurale (IER)″ at the ″Centre Regional de Recherche Agronomique (CRRA)″ of Sikasso. Rice plant samples were collected from both leaf infections. Besides rice, samples were also obtained soil samples from rice growing fields in Sikasso.

Isolation of pathogen

Blast lesions were surface sterilized with 0.1% mercuric chloride for 1 min, washed with sterilize distilled water and placed over clean glass slides kept in sterile Petri dishes padded with moist cotton. The Petri dishes were incubated for 48 h at room temperature (28 ± 2°C). Single spore method was used for purification (Noman et al., 2018; Zhang et al., 2013). For that, single conidia were identified from the sporulating lesions using a stereomicroscope and aseptically transferred to a petri dish (PDA media). The isolated and purified strain was identified mainly from its macroscopic characters of its colonies and microscopic characters of its mycelium on the basis of identification keys (Botton et al., 1990).

Media suitable for culturing *P. oryzae*

The determination of suitable media for culturing *P. oryzae* (*M. oryzea*) was done according to the method described by Vanaraj et al. (2013). The *M. oryzae* Longorola strain (*M. oryzae* LS) was grown on PDA for 10 days at room temperature. From the margin of actively growing fungus, 5 mm discs were plugged out. Sterile Petri dishes containing PDA, oat meal agar, rice agar, rice polish agar and malt extract agar were inoculated each with a single 5 mm disc of the fungus and incubated at room temperature for 30 days. Four replications were maintained for each medium. The fungal growth was measured at 5-day-intervals until 30 days. Further, the colony characters of the single isolate on different media were recorded on 30th day. All the 11 isolates were grown on PDA and their colony morphology was observed.

Spore induction on stem bits

As the fungus grows and sporulates slowly on rice, but sporulate more quickly on maize and *P. ripens* and we need more spores for the pathogenicity test; we tested the spore production ability of the fungus on these plants. For that, the stem bits from maize, rice (20-day old crop) and *P. repens* were collected from the field and cut into small pieces of 1 cm in length. 15 pieces were placed in 50 ml Erlenmeyer flasks and sterilized for 1 h and 30 min. Each flask was inoculated with two 5 mm diameter mycelial discs of the Longorola isolate and incubated for 15 days at room temperature (Vanaraj et al., 2013). Three stem pieces were sampled at 5, 10 and 15 days after inoculation (DAI). Each stem piece was placed in a test tube containing 1 ml of sterile water, shaken well to dislodge the spores and decanted. The spore concentration was assessed using a haemocytometer.

Measurement of spore size

After spore induction on bits tests shows that the fungus grows and sporulates more quickly on maize bits than on rice and *P. repens*. The *M. oryzae* LS isolate was multiplied on maize stem bits for 15 days and spores were collected by placing stem piece in a test tube containing 1 ml of sterile water, shaking well to dislodge the spores and decanting. The length and width of 10 spores were measured for each isolate using a micrometer as in Vanaraj et al. (2013).

Isolation and identification of *Trichoderma* sp.

One gram of the soil sample was taken and added to 1 ml of sterilized distilled water to make a dilution of 10^{-1}. This suspension was then subjected to serial dilutions and a dilution of 10^{-5} was attained. One milliliter of each dilution viz., 10^{-3} to 10^{-4} was poured on to *Trichoderma* Specific Medium (TSM) (Elad et al., 1981) and purified by single spore method (Zhang et al., 2013). The TSM medium is composed of the following constituents (g/L): $MgSO_4$ 9; 7 H_2O, 0.2; K_2HPO_4, 0.9; KCl, 0.15; NH_4NO_3, 1.0; glucose, 3.0; chloramphenicol 0.25; *p-dimethylaminobenzenediazo* sodium sulfonate 0.3; pentachloronitrobenzene 0.2; rose-bengal 0.15; agar 20 Isolates were identified on the basis of their morphological characters, according to conidiophore, shape of the phialides and emergence of phialophores and phialospores (Soesanto et al., 2011). The purified and identified cultures of *Trichoderma* strains were maintained on PDA medium and stored at 4°C for further use.

Antagonism characterization

Out of 12 isolates of Trichoderma sp. obtained from different soil samples, only 3 strains showed, in pre-test, an antagonistic activity against the rice blast pathogen isolated. These three isolates identified as *T. harzianum* were tested for antagonism effect against the isolated rice pathogen: *M. oryzae*. The antagonism studies were done by using dual culture techniques as developed by Rahman et al. (2009). The mycelial bits of 5 mm diameter of *Trichoderma* sp. strain and pathogen were placed opposite to each other (4 cm) on Petri plates containing PDA. The plates were run in triplicates with one control set maintained without inoculating the *Trichoderma sp.* isolates. The plates were incubated at 28 ± 2°C for one week, and the growth of the pathogen tested against the 3 isolates of *Trichoderma* which showed antagonism activity in the pre-test. The data were recorded regularly on the growth of the pathogen and *Trichoderma* sp. tested. Percentage of mycelial growth inhibition (MGI) was calculated according to the formula:

MGI% = (dc - dt) ×100/dc

Where, dc= fungal colony diameter in control sets, dt= fungal colony diameter in treatment sets.

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Plates were tested in triplicate, with a control set maintained without inoculating Trichoderma sp. The plates were incubated at 27+-1°C for one week, and pathogen growth was tested against the 3 Trichoderma isolates that showed antagonistic activity in the pre-test. Data was recorded regularly on the growth of the pathogen and Trichoderma sp. Tested. The percentage inhibition of mycelial growth was calculated according to the formula:

MGI%=(dc-dt)×100/dc

Where, dc=diameter of fungal colony in control sets, dt=diameter of fungal colony in treatment sets.

The inhibition exerted by the genus Trichoderma was estimated by calculating the antagonism coefficient (Morsy, 2005) according to the following formula:

$a=(C_{tem}-C_{trait})/C_{tem}$

where a is the antagonism coefficient, Ctem the mean radius of the control colonies (strains of phytopathogenic fungi growing in the absence of antagonist), C_{trait} the mean radius of the colonies in the presence of the antagonist.

The evaluation of myco-parasitic activity was carried out in accordance with the method of Dabire et al. (2016). This method consisted in carrying out cocultures in direct confrontation between the pathogen and the antagonist for 21 days. After the 21 days, mycelial fragments of the pathogen in the area of intersection were cultured at 25°C for 5 days to observe the viability of the pathogen. Sandwiched Petri plates, a setup described in Li et al. (2018), was employed to determine if the *Trichoderm*a isolates tested can produce volatile compounds, and how the volatile compounds affect the growth of *M. oryzae* LS. After inoculating *M. oryzae* LS and *Trichoderma* isolates on PDA plate, *M. oryzae* LS plate was placed on top of *Trichoderma* plate, sealed with three layers of Parafilm, and incubated at 25°C. Each plate of *M. oryzae* LS also was sandwiched with an un-inoculated PDA plate (control treatment). Colony diameter of *M. oryzae* LS was measured 5 days later. We evaluated the inhibitory effect of *M. oryzae* LS volatile compounds on *Trichoderma* in the same way except that 5-day-old (after the inoculation of culture plug) *M. oryzae* LS culture was used to ensure enough *M. oryzae* LS biomass. Colony diameter of *Trichoderma* was measured 36 h later. The duration of volatile compound exposure between the two experiments was different because *Trichoderma* grew much faster than *M. oryzae* LS. Each treatment included three biological replicates and was repeated three times.

RESULTS AND DISCUSSION

Virulent strains of *M. oryzae*

In this study, only one isolate of *M. oryzae* (Figure 1) was obtained from a sample of rice from the Longorola research station of the CRRA in Sikasso (IER), and named *M. oryzae* Longorola strain (*M. oryzae* LS).

Growth of *M. oryzae* was rapid on PDA followed by malt extract agar (Table 1 and Figure 1). At 5 days after inoculation (DAI), the colony diameter was 3.45 cm. The *M. oryzae* isolate grew 8.95 cm in diameter in the PDA, contained in 9.0 cm Petri dishes, at 10 DAI. On the same day, its growth on the rice agar was 6.95 cm. At 15 DAI, the colony diameter of *M. oryzae* was 9.0 cm in both media tested.

The spore density was greatest when *M. oryzae* was propagated on maize followed by the rice stalk at all observation intervals (Table 1). The production of conidia increased over time for both maize and rice stalks. For maize, it was respectively 395, 1630, 2900 at 5, 10 and 15 DAI. This *M. oryzae* strain produce very few spores on *P. repens*, a grass which was found support growth and bigger spores of *M. oryzae* than rice. As maize stalk shows better grow and more spore production, we chose to multiply *M. oryzae* on maize stalk to have more spore in a relatively short time for tests.

The length and width of *M. oryzae* conidia (Figure 1)

Figure 1. Mycelia and spores of *M. oryzae*.

Table 1. Assessment of spore density on maize and rice stalks.

Media	Number of spores/ml of water		
	Days after incubation (DAI)		
	5	10	15
Maize	395	1630	2900
Rice	90	320	600
Panicum repens (*P. repens*)	43	109	200

Table 2. Length and width of the strain of *Magnaporthe oryzae* LS, isolated from Longorola (Sikasso), Mali.

Isolate	Length		Width	
	Interval	Average	Interval	Average
M. oryzae LS	20 - 23	21.5	8.95- 10.25	9.6

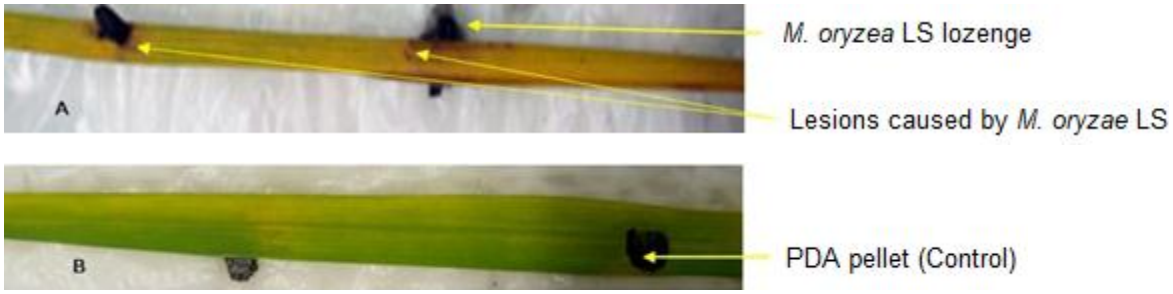

Figure 2. Lesions caused by *M. oryzae* LS pellets (A), while PDA pelletsdid not cause any damage (B).

were measured. Results of spore dimension measurement, showed that the mean spore length of the *M. oryzae* isolate was 21.5 µm, while the mean spore width was 9.6 µm (Table 2).

Virulence of *Magnaporthe oryzae* LS strain on rice

The strain of *M. oryzae* LS inoculated on the rice leaves, kept under humidity, showed lesions similar to those of blast (Figure 2A). The uninoculated control, meanwhile, showed no signs of pathology (Figure 2B).From the lesions of the inoculated rice leaves, we were able to re-isolate the *M. oryzae* LS strain. These results confirm the pathogenicity of the isolated *M. oryzae* LS strain.

Strains of *Trichoderma* sp. isolated and identified

Isolation of *M. oryzae* antagonist fungi from soil and rice

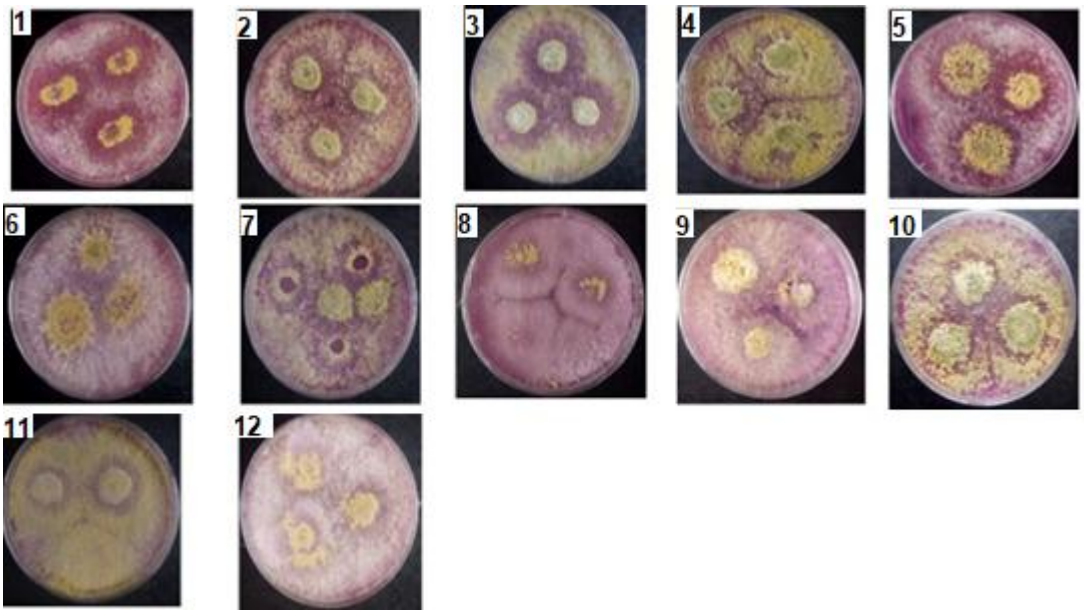

Figure 3. Macroscopic characteristics of the 12 *Trichoderma* isolates analyzed. These isolates are from analyzed soil samples of rice fields in Sikasso, Mali.

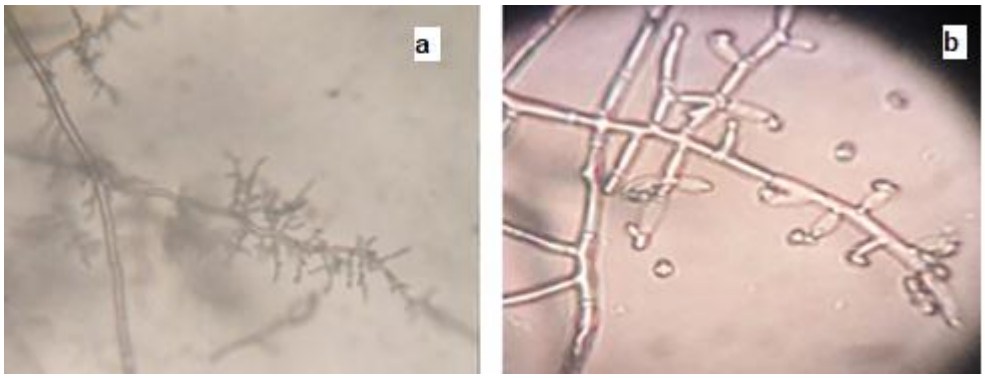

Figure 4. Conidiophore and conidia of *T. harzianum* isolates from soil 1 (a) and 3 (b).

plant samples yielded 12 colonies (Figure 3). These isolates gave colonies typical of *Trichoderma* sp. on *Trichoderma* selective medium. These isolates showed very rapid growth, good sporulation, and yellowish-green and green pigmentations on PDA medium. Three isolates from soil 3 (S31, S32 and S33) (Colonies number 1, 2 and 3 in Figure 3) showed very rapid growth and formed 2 concentric rings with green conidial production. The conidia production was denser in center then towards the margins on PDA medium. The mycelia of these 3 isolates are septated. For these 3 isolates, the conidiophore is sparsely branched and carries philaids (Figure 4). In turn, phialides carry sub-globular-shaped spores or conidia. On the basis of their cultural and morphological characters of these three *Trichoderma* isolates, were identified as *T. harzianum* (Gams and Bisset, 1998; Shah et al., 2012. These 3 strains, that showed, in a pre-test, an antagonistic activity against *M. oryzae* LS) are under molecular characterization to confirm the identity of each.

Antagonism tests

The average growth ranges of *M. oryzae* LS placed in cocultures in direct confrontation with the isolates of *T. harzianum* are given in Table 3. Analysis of data in Table 3 indicates that all of the *T. harzianum* isolates caused a significant reduction in the average growth radius of the *M. oryzae* LS strain tested. The highest coefficient of antagonism obtained was 0.78 obtained with the strain *T. harzianum* S33 (Table 3), followed by the strain *T.*

Table 3. Radial growth and coefficient of antagonism of 3 isolates of *T. harzianum* on *M. oryzae* LS.

Treatments	*M. oryzae*	
	Growth (cm)	Coefficient of antagonism
M. oryzae LS	4.5	-
M. oryzae LS + *T. harzianum S3*1	2	0.55
M. oryzae LS + *T. harzianum S3*2	1.3	0.71
M. oryzae LS + *T. harzianum S3*3	1	0.78

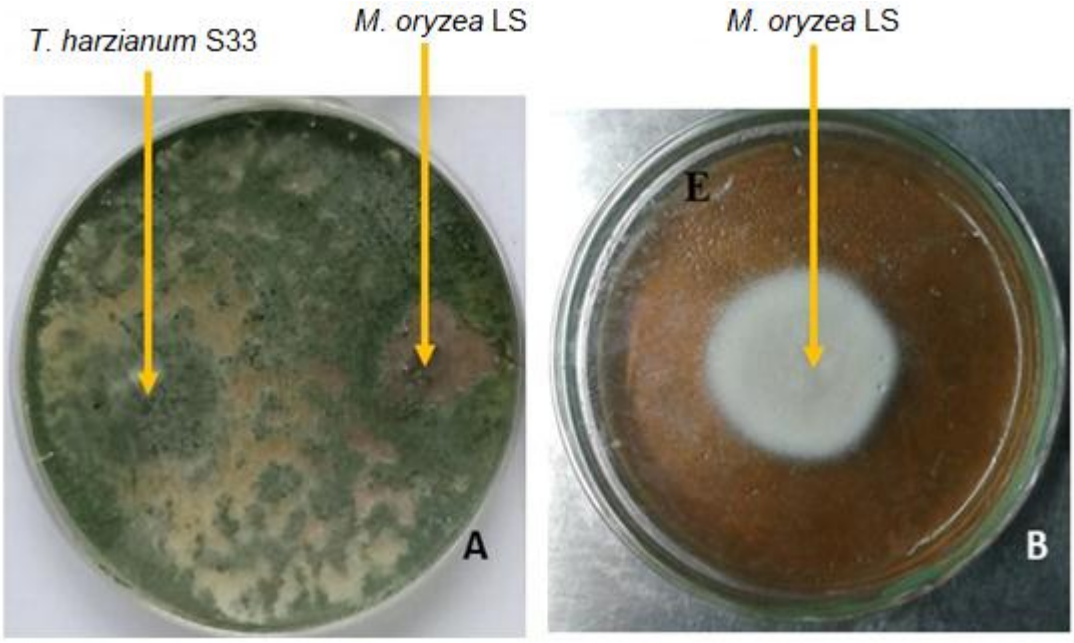

Figure 5. Effect of *T. harzianum S3*3 on the mycelium growth of *M. oryzae LS* (A) strain and the growth of the *M. oryzae* strain only (B).

harzianum S32 with a coefficient of antagonism of 0.71. The *T. harzianum* S31 strain gave the lowest coefficient of antagonism estimated at 0.55. The effect of *T. harzianum* S33 on the mycelium growth of *M. oryzae* LS is presented in Figure 5. The results on mycoparasitism presented showed that only the *T. harzianum* S33 demonstrates a clear mycoparasitic activity against *M. oryzae* LS (Table 4). None of the three *Trichoderma* strains tested were able to control the growth of the *M. oryzae* LS strain by producing volatile compounds.

DISCUSSION

Twelve different colonies of *Trichoderma* sp. were isolated from soil samples from the Longorola station and Sikasso rice fields. Küçük and Vanç (2003) isolated 19 strains of *Trichoderma* from 31 soil samples, while we isolated 12 from 5 sample from agricultural soil of Sikasso (Mali). Which is an indicator of the richness of these soils compared to those of the Turky? Growing the fungus on pieces of the host's stem is the easiest way to induce sporulation in order to study spores (Vanaraj et al., 2013). *M. oryzae* spores isolated from rice in this study were significantly smaller than those isolated from the rice fields of Baguineda. Gupta et al. (2020) states that the spore size of the fungus *M. oryzae* varies among isolates depending on environmental conditions. Measurement of the spore size of different isolates of *M. oryzae* from rice grown on PDA by Gayatonde et al. (2016) showed that the mean length ranged from 21.2 to 28.4 µm. In our study, the spore length of *M. oryzae* isolates grown on pieces of corn stalk was 21.5. Vanaraj et al. (2013) observed variations in the length and width of *M. oryzae* spores due to the effect of artificial media. They also noticed that temperature had no effect on the width of the spore while the length was affected.

The results obtained at the end of this study reveal that

Table 4. Mycoparasitic activity of *T. harzianum* S31, *T. harzianum* S32 and *T. harzianum* S33 strain on *M. oryzae* LS.

Pathogenic	Antagonists		
	***T. harzianum* S31**	***T. harzianum* S32**	***T. harzianum* S33**
M. oryzae LS - Exp 1*	**-***	**-**	**+**
M. oryzae LS - Exp 2	**-**	**-**	**+**
M. oryzae LS - Exp 3	**-**	**-**	**+**

Exp 1, Exp 2 and Exp 3 represent respectively Experiment 1, Experiment 2 and Experiment 3
*Result from 3 replicates.

out of all the *Trichoderma* isolated in Mali, only 3 have an inhibitory activity on the mycelial growth of *M. oryzae*, but to different degrees. Naravanasamv et al. (2015) demonstrated the inhibitory activity of *Trichoderma* species on the growth of *P. oryzae*. In this study, in addition to antifungal activity, only the *T. harzianum* S33 strain exhibited myco-parasitic activity on the *M. oryzae* isolate tested. In addition, an antagonism coefficient of 0.71 was obtained with this strain. These results are also in the same direction as those of Li et al. (2018) who showed that 5 isolates of *T. harzianum* tested have a strong inhibitory power against 3 pathogenic fungi, including *Fusarium oxysporum f*.sp. *cepae* (82.77%). However, not all of the 5 isolates exerted a myco-parasitic action on all the pathogens tested and the intensity of the inhibitions varied from one pathogen to another and from one antagonist to another but values antagonism coefficients greater than 0.75 were obtained with two of the isolates tested. The antagonism in the distance confrontation was much lower than in the direct confrontation. This could be explained by the fact that *T. harzianum* uses several modes of action and depending on the nature of the pathogen to exert their antagonistic power (Benitez et al., 2004; Sood et al., 2020). According to Benhamou and Chet (1997) fungi chitin is an essential constituent of the wall which surrounds and protects cells from the environment. In the same way, Latgé (2007) showed that the cell wall is essential for fungal growth and for the resistance of the fungus to external attacks. Its alteration linked to the action of *Trichoderma* would lead to an alteration of the mycelium which results in aggregation, retraction and vacuolation of the cytoplasm. The statements by Benhamou and Chet (1996) and Latgé (2007) were confirmed by Tapwal et al. (2015), who indicate that the inhibitory power of *Trichoderma* species is manifested by a significant lysis of the mycelial cells of the pathogens. Nusaibah and Musa (2019), reports that *Trichoderma* species have the ability to attack pathogens via different modes of action. They can use the antibiotic which results from the production of substances which act as "antibiotics" and which inhibit the growth of the pathogen. In some cases, the colony of *T. harzianum* grows on that of the pathogen. According to Sharma (2011), these types of interactions indicate competition and parasitism, respectively. The results obtained in remote confrontation mode indicate that the use of volatile compounds was not the main path that explains the observed antagonism.

Conclusion

Only one pathogenic isolate of *M. oryzae* was isolated from soil and diseased rice plant samples from Sikasso, Mali. Twelve isolates of *Trichoderma* were isolated from soils samples. Out of these isolates: *T. harzianum* S31, *T. harzianum* S32 and *T. harzianum* S33 showed high antagonism activity against *M. oryzae* LS isolated from Mali.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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