Int.J.Curr.Microbiol.App.Sci (2024) *13*(06): 252-259

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 13 Number 6 (2024)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2024.1306.027

Invitro Evaluation of *Trichoderma* Strains Against *Pyricularia oryzae*, the Causal Agent of Rice Blast Disease

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Keywords

Inhibition, Mycelial growth, Trichoderma strains, *Pyricularia oryzae*, biocontrol

Article Info

Received:
28 April 2024
Accepted:
31 May 2024
Available Online:
10 June 2024

A B S T R A C T

Phytopthogen are simply an organism parasitic on a plant host resulting the serious problems regarding crop losses in agriculture sector. To facing such a threat, it is very wise to use Biological Control Agents (BCA's) like *Trichoderma*, a soil-borne filamentous fungus that are capable of parasitizing several plant pathogenic fungi. It is a potential fungal BCA's against a range of plant pest and pathogens. Unfortunately, popularization of bio-pesticides is very slow as compared to chemicals. In this study, *Pyricularia oryzae* was isolated from diseased plant parts using tissue culture techniques. Thereafter, antagonistics properties of two T-strains coded as Ts7B1 and Ts8O were evaluated against *P.oryzae* isolate tested in vitro study using dual culture technique. In dual culture techniques it is found that Ts7B1 has maximum mycelial growth inhibition and sporulation of pathogen (as much as 60 and 69% respectively) whereas Ts8O has lowest effect on it (35 and 55.2%). Present study concludes the uses of *Trichoderma* and assessment of their suitability as bio-pesticides for control of P.oryzae, the causal agent of blast rice disease.

Introduction

Rice (*Oryzae sativa* L.) is one of the main cereal food crops in most part of Africa. The importance of rice has led to increasing country-specific, regional, and multinational efforts to develop germplasm and policy initiatives to boost production for a more food-secure continent. It is recognized as an important strategic food security crop and as a crucial element in the staple food economies of Sub Saharan Africa (Abdu *et al.*, 2013). The rice average yield in Democratic Republic of Congo (DRC) is 0.8 tons/ha in upland rain-fed and 1.2 tons/ha in lowland areas (MINAGRI, 2016). Currently, this critically important cereal crop is predominantly cultivated by small-scale farmers under suboptimal conditions in most parts of DRC.

The major problems in rice production around the world are biotic and abiotic stresses against rice crops (Ou, 1985). Blast disease is caused by a filamentous, ascomycetous fungus, *Magnaporte grisea* (Hebert) Barr., anamorph *Pyricularia oryzae* (Mebratu *et al.,* 2015) is an important fungal disease of rice known to occur in most rice producing areas of the world (Ou, 1985). The fungal rice blast disease infects all above ground parts of the plant but the leaf and panicle lesions are the most serious (Zeigler and Correa, 2000). The major control measure includes use of fungicides and resistant varieties. Fungicides are costly, harmful to the environment and sometimes have low efficacy (Bonman, 1992).

Resistance is a viable method of control as long as it lasts. Experience has shown that elite varieties succumb to rice blast disease within few years (Kariaga *et al.,* 2016). It is not known whether it is the pathogen developing ability to overcome resistance to cultivars or the frequency to genetic changes of formerly rare pathotypes to new virulent forms (Srivastava *et al.,* 2014; Mebratu *et al.,* 2015; Kariaga *et al.,* 2016). Differentially accumulated proteins were identified in mycelia of several *Pyricularia oryzae* isolates showing different virulence on the rice plants to reveal the *Pyricularia oryzae* fungal pathogenesis and to suggest novel disease control method.

Biological control is an alternative approach for disease management that is environmentally safe and reduces the amount of human contact with harmful chemicals and their residues. A variety of Biological Control Agents (BCAs), including fungi and bacteria, have been identified but further development and deployment is required (Muhammad *et al.,* 2022; Sharma *et al.,* 2017). The limited availability of commercial BCAs has been a major constraint to the development of eco-friendly and sustainable disease management worldwide (Vincent *et al.,* 2007; Villaverde *et al.,* 2016). The key factor in developing effective and efficient BCAs is the exploration for potential BCAs across agricultural production regions globally. Therefore, isolation, screening and identification of local BCAs are needed, including in DRC.

The use of microorganisms in biological control has many advantages including easy dissemination, specificity of their action on certain target organisms, effectiveness at low initial administration doses as well as persistence and ubiquity in the pathosystem. A melampolide, called sonchifolin, isolated from foliar extracts of *Smallanthus sonchifolius*, showed strong inhibitory activity against *P.oryzae* (Inoue *et al.,* 1995). Alaya Ben Salem (2013) demonstrated the inhibitory capacity of strains 7B1 and 8O of *Trichoderma sulphureum* on *Aspergillus niger* and *Fusarium solani*in vitro in direct confrontation.

Being part of this concern and having in mind its implication in the specifically Congolese context. This has led to research on alternative strategies for controlling *P.oryzae* on rice. Interest in biological control of rice blast disease has increased over recent decades (Saharan and Mehta, 2008).

The present study aims to evaluate the antagonistic potentiality of *Trichoderma* strains as Biological Control Agents against *P. oryzae* on the two life stages: mycelial growth and sporulation.

Materials and Methods

Isolation of Plant Pathogenic Fungi

The diseased plant samples showing typical blast symptoms on rice leaves were collected from the field and brought to the laboratory, washed repeatedly with tap water. Thereafter, small pieces of diseased portion were cut using sterilized blade for isolation. Care was taken that each cut piece should have some healthy parts as well. The pieces were then surface sterilized in mercuric chloride (HgCl2) solution (1:1000) for 20- 30 sec. followed by thorough rinsing in sterilized distilled water, thrice. The surface sterilized pieces were then aseptically transferred separately to the plates containing PDA medium and then incubated at 25 ± 2°C. After 48-72 h of incubation, the growing mycelium from the margin of apparently distinct colonies was sub-cultured on fresh PDA slants. In this way, the culture of *P.oryzae* was isolated.

Biological Control agents (BCAs)

Two strains of *Trichoderma sulphureum* were tested for their antagonistic effect in dual culture tests: Ts7B1 and Ts8O. The strains used were obtained from the collection of the Kinshasa Plant Clinic laboratory and stored at 4°C. The fungal cultures are incubated at 25°C and in the dark for one 7 days before use. For dual culture tests, one inoculation method was used for the pathogen, either mycelial plugs.

Chemical Treatment

A fungicide belonging to the chemical family of Benzimidazoles was used in this study as a control. This is Benlate®. The choice of Benlate® was based on its fungitoxic effectiveness demonstrated by Serghat *et al.,* (2004) and its registration status in the country and its availability.

Invitro conidial production and mycelial growth of *P.oryzae*

Mycelial and colony growth

A 3-day-old on PDA grown, 5 mm of mycelial disc from young cultures of *P.oryzae* was placed in the centre of each of 3 Petri dishes and then incubated in growth room at 25°C in the dark. Three replicates were prepared for each pathogen-Treatment combination.

Radial mycelial growth was measured every day until day 8 which corresponds to the total occupation of the Petri dish. This was done to calculate the daily rate (cm/day).

Sporulation

For the assessment of sporulation, tubes containing four rounds of 5 mm diameter, taken along the diameter of the *P.oryzae* colony (8 days old) were used in 1 ml of 'distilled water. The fungal suspension is then shaken using a vortex for 20 seconds to release the spores from the conidiophores. Sporulation of the fungus was evaluated using the direct method of counting using a hemacytometer or Thoma cell under a light microscope. Values are expressed as number of spores per unit area (mm^2) and these experiments are repeated three times.

In vitro biological control of *P.oryzae* by potential BCAs (inhibition of mycelial growth and sporulation)

Dual culture method of BCAs

Antagonistic activity of *T.sulfureum* strains against *P.oryzae* isolate was determined through dual culture technique (Dennis and Webster, 1971). Petri dishes (90 mm) containing PDA were inoculated with a 5 mm disc of 7 day old pure culture of antagonistic fungi and pathogen (Fig.1). One mycelial disc of fungus was placed at opposite sides on PDA plates and incubated at 28 °C on darkness in completely randomized design (CRD). Control Petri plates were inoculated with *P.oryzae* and a sterile agar plug. A number of three replications were assigned to each treatment. The experiment was repeated once. Growth of *P.oryzae* isolate in dual culture and in control (without antagonist) was measured after different intervals i.e. 1, 2, 3, 4 and 5 DAI (Days After Incubation) and percent inhibition of radial growth was determined with following formula:

$$Inhibition\ percentage\ of\ radial\ mycelial\ growth\ (Ic) = \left[\frac{(C - T)}{C}\right] x\ 100$$

Where C is the pathogen radial mycelial growth measurement in control plates, and T is the pathogen radial mycelial growth in presence of *T.sulfureum* strain (Simonetti *et al.,* 2012).

Reduction in the sporulation (I_s) was calculated as follows (14):

$$Inhibition\ of\ sporulation\ (Is) = \left[\frac{(S - T)}{S}\right] x\ 100$$

Where S is the pathogen sporulation in control plates, and T is the pathogen sporulation in presence of *T.sulfureum* strains and chemical treatment (Simonetti *et al.,* 2012).

For analysing fungal mycelial growth affected by the chemical treatment, mycelial discs (5 mm in diameter) were cut from growing edge of 7-day-old fungal colonies and placed upside down at the center of 1/2-strength PDA supplemented with a concentration of the chemical. Colony diameters of *P.oryzae* were measured 7 days after culture at 25°C under darkness. Relative fungal colony diameters by the chemical treatments were expressed as percentage (%) compared to that in the untreated control.

An analysis of variance (ANOVA) was conducted to determine the effects of treatments with chemicals on the fungal growth. Means were compared using least significant difference test. Statistical analysis was performed with the STATSTIX version 8.0.

Results and Discussion

Radial mycelial growth

Examination of the radial growth rate (Fig.2), calculated on the linear phase of the evolution of the growth curve, shows the differences between strains with controls that appear after the 2nd dateuntil the 7th day after confrontation which corresponds to the total invasion of the petri dish.

There were significant differences (*P≤0.001*) in growth rate among treatments at 2DAI and 5DAI of incubation. At 2 DAI, chemical treatment (Benlate®) had the smallest radial mycelial growth with diameters of 1.1 cm (2 DAI) and 1.4 cm (5 DAI), followed by the Po-Ts7B1(1.6 cm at 2 DAI, 3.2 cm at 5 DAI) and Po-Ts8O (2.6 cm at 2 DAI, 5.1 cm at 5 DAI). The Control (Pathogen only) had the highest radial mycelial growth at 2 DAI (2.2 cm) but had accelerated growth at 5 DAI (8 cm).

Sporulation

Examining the results of figure 3 shows a large difference (*P≤0.001*) among treatments. Strong production is observed in control (2900 conidia). Low production is recorded in the Benlate® (200 conidia). Po-Ts7B1 and Po-Ts8O showed medium production ranging from 900-1300 conidia.

In vitro biological control of *P.oryzae* by potential BCAs

All Trichoderma strains showed some capacity to reduce mycelial growth and the number of conidia of *P. oryzae*in dual culture tests in Petri dishes (Table 2 and figure 4).

There were significant differences (*P≤0.001*) in inhibition of both radial mycelial growth and conidia production by the pathogen among the treatments. Mycelial growth was inhibited by 35–82.5%. The highest inhibition was caused by P-Fc (82.5%), follows by Po-Ts7B1 (60 %) and Po-Ts8O (35%). The presence of fungicide decreased conidia production by 93.1%. P-Ts.7B1 inhibited the production of conidia by the pathogen (69%), while P-Ts.8O had the least potential to inhibit conidia production, reducing the number of conidia by 55.2%.

The application of biological control agents is a promising tool to manage the damage caused by plant pathogens. Biological control treatments for plant pathogens must provide enhanced levels of disease suppression and consistency of control over diverse soils before their wide-scale application on a commercial scale (Hu *et al.,* 2019).

The results of the dual culture method show that *Trichoderma* strains can significantly reduce mycelial radial growth and sporulation density (Seema and Devaki, 2012). Indeed, macroscopic observation shows that the two *Trichoderma* strains tested inhibited the mycelial growth of the pathogen and developed on the surface of *P.oryzae* colonies and microscopic examination shows similar results concerning the production of conidia to those obtained by Barakat *et al.,* (2007) and Hidayah *et al.,* (2022).

Furthermore, some *Trichoderma* species can induce host resistance responses against pathogens (Harman *et al.,* 1996). Several studies have shown the biocontrol potential of *Trichoderma* species in controlling pathogens in *in vitro* and *in vivo* conditions (Ojaghian, 2011; Alaya Ben Salem *et al.,* 2013). For example, an *in vivo* seed coating test using thiophanate-methyl or *Trichoderma* spp. substantially improved soybean germination and suppressed growth of *S. sclerotiorum* (Macena *et al.,* 2020). Our results are consistent with previous research where colonies of *T. longibrachiatum, T. atroviride* and *T. harzianum* grew faster than *S. sclerotiorum* in both single or mixed cultures (Matroudi and Zamani, 2009). Our studies also showed that strainTs7B1were highly effective in reducing mycelial growth and completely inhibiting conidia production by the pathogen as also reported by Gupta *et al.,* (2014). Furthermore, Ts8Ohas been shown to reduce colony growth and conidia production by 35 and 55.2%. Knowledge on the effectiveness of the new strains of BCAs on controlling the critical stages of the life cycle of a particular pathogen is very important to determine the most effective strains for commercialization.

Table.1 Details of treatments for in vitro experiment

N°	Treatment	Code
1	Pathogen only (Untreated Control)	Control
2	Pathogen + Fungicide (Benlate®)	P-Fc
3	Pathogen + Ts7B1	P-Ts7B1
4	Pathogen + Ts8O	P-Ts8O

Table.2 Inhibition percentage of mycelial growth and sporulation

Treatments	Inhibition percentage (%)	
	Mycelial growth	Sporulation
P-Fc	82,5	93,1
P-Ts.7B1	60	69
P-Ts.8O	35	55,2

Figure.1 Growth rate on PDA of the isolate of *Pyricularia oryzae* in dual culture with BCAs

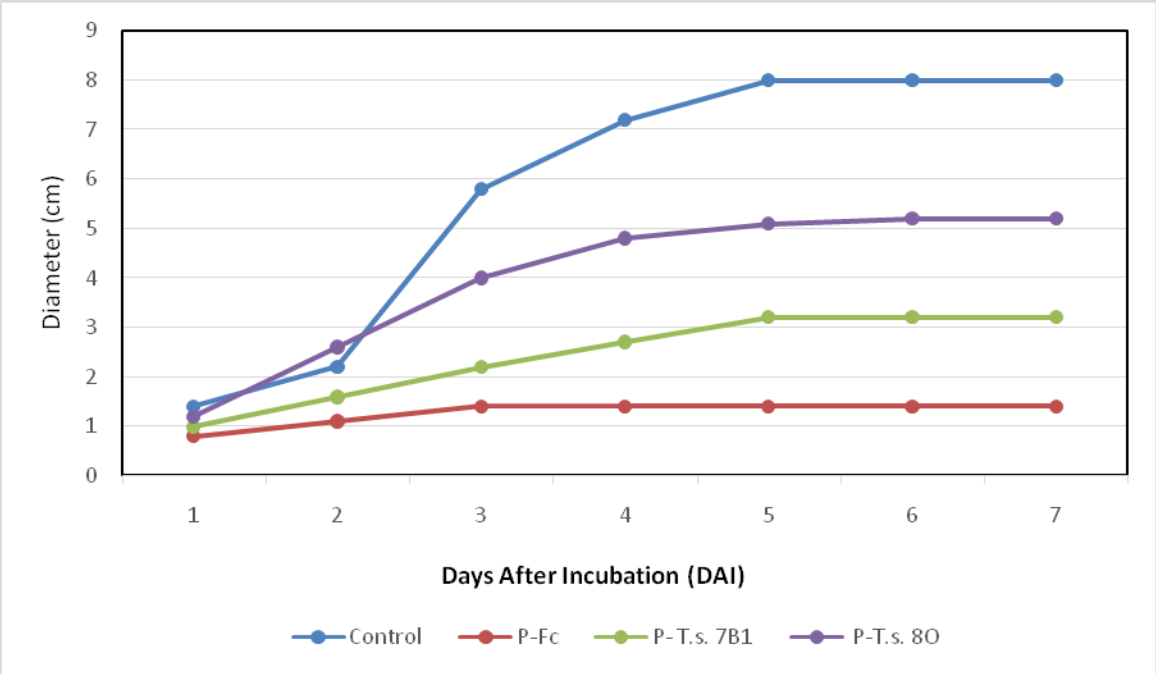
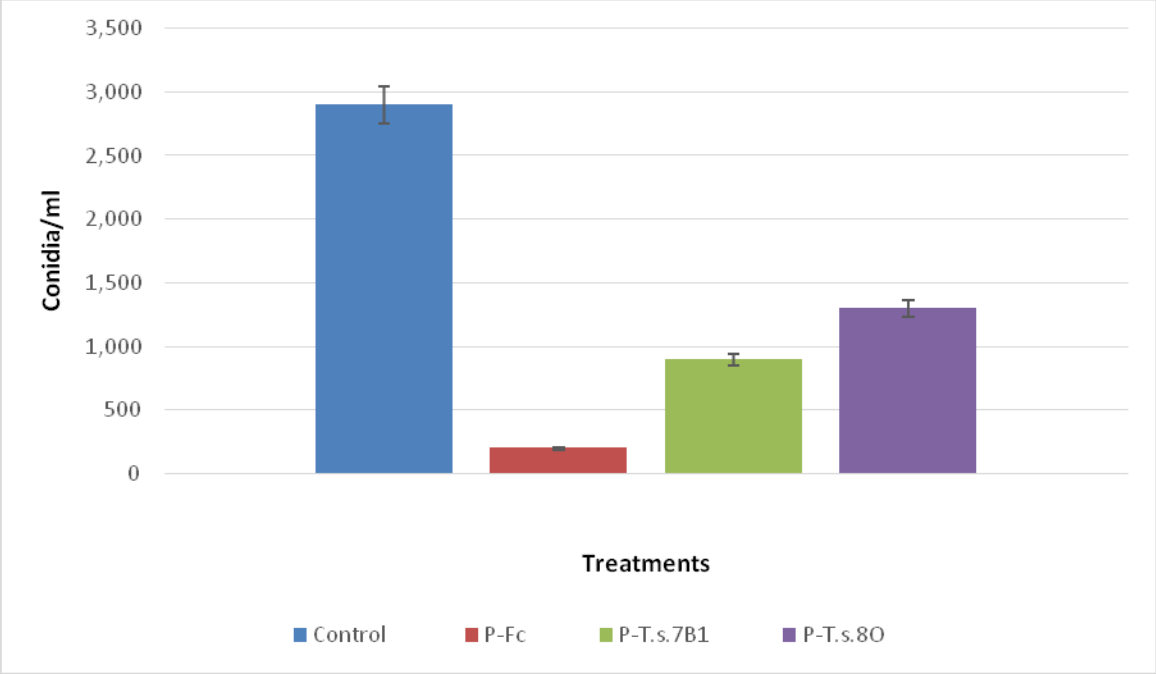


Figure.2 Production of conidia of *P.oryzae* in dual culture with BCAs7DAI



Our *in vitro* experiments revealed significant inhibition of conidia production by *Trichoderma* strains in dual plate inoculation treatments. The nearly complete inhibition of conidia production could possibly be due to reduced viability of mycelia. It could also be attributed to competition for space and nutrients or mycoparasitism reducing growth and consequently inhibiting the conidia production ability of the pathogen. Our results corroborate those of Abdullah *et al.,* (2008) who reported that *Trichoderma* species had an ability to control both mycelial growth and conidia production by *P.oryzae* when tested on the same plate.

Eco-friendly disease/pest resistance strategies are modern concern in modern and sustainable agriculture. Bio-control agents have emerged as new strategies of managing plant disease by inducing systematic resistance plus growth promotion in plants against diseases. Biocontrol is an alternative, eco-friendly means for managing plant diseases/pest. Restriction on pesticides use and widespread emergence of pathogen resistance has increased global demand of biopesticides. It can be concluded from the present studies that *Trichoderma* strains can serve as good option for biocontrol against *P.oryzae* based on its inhibition percentage in vitro experiment. These facts are concluded on the basis of following results found on this study:

- Strain Ts7B1 should be preferred over other tested strain for mycelial growth and conidia production.
- The present study provides preliminary information on the antagonistics properties of *Trichoderma*. Out of the two *Trichoderma* strains, Ts7B1 showed strong antagonistic activity against *P.oryzae*.
- *Trichoderma* is a potent BCA's and used extensively for pre/post-harvest disease control; used successfully against *P.oryzae*.

These facts are supported by present study where result sections shows positive values of inhibition percent diseases index increase over control due to various mechanisms involving *Trichoderma* phytopathogen interaction described in discussion section.

Author Contribution

L. Tshilenge-Lukanda: Investigation, formal analysis, writing—original draft. A. Ngombo-Nzokwani: Validation, methodology, writing—reviewing. J. Mukendi:—Formal analysis, writing—review and editing. M. Muengula-Manyi: Investigation, writing—reviewing. J. Mudibu: Resources, investigation writing—reviewing. A. Kalonji-Mbuyi: Validation, formal analysis, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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How to cite this article:

Tshilenge-Lukanda, L., A. Ngombo-Nzokwani, J. Mukendi, M. Muengula-Manyi, J. Mudibu and Kalonji-Mbuyi, A. 2024. *In vitro* Evaluation of Trichoderma Strains Against *Pyricularia oryzae*, the Causal Agent of Rice Blast Disease. *Int.J.Curr.Microbiol.App.Sci.* 13(6): 252-259. **doi:** https://doi.org/10.20546/ijcmas.2024.1306.027