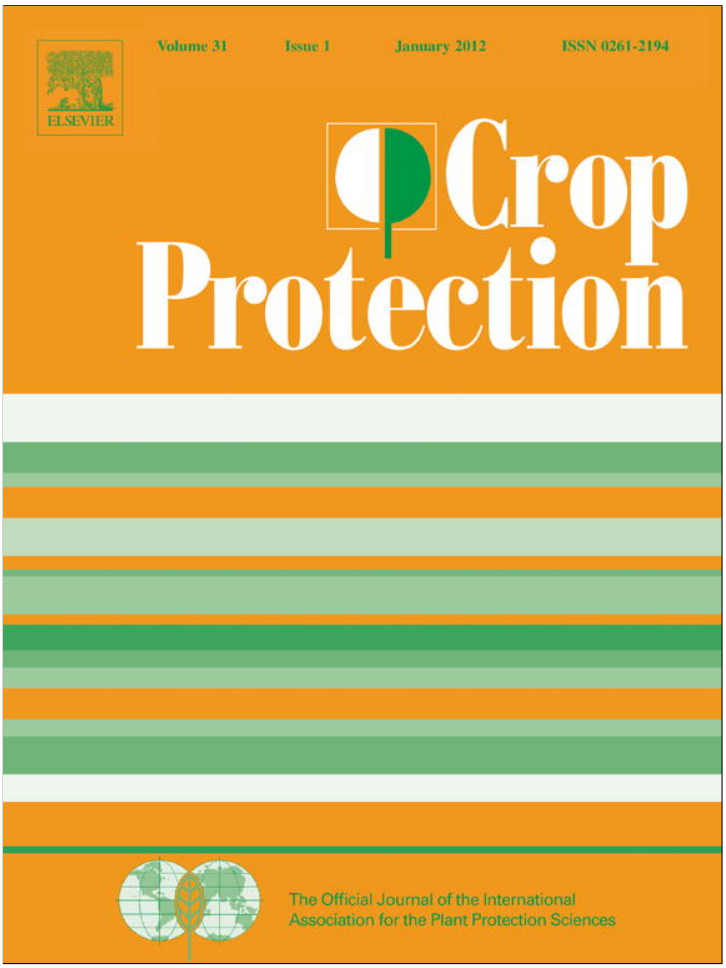


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Short communication

# *Trichoderma asperellum:* A potential biocontrol agent for *Pythium myriotylum*, causal agent of cocoyam (*Xanthosoma sagittifolium*) root rot disease in Cameroon

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ABSTRACT

Root rot disease caused by *Pythium myriotylum* is a major threat to cocoyam cultivation in Cameroon. Control methods based on agricultural practices and chemical treatments have limitations. Since biocontrol is an interesting alternative, this study assessed the potential of four *Trichoderma asperellum* strains to control *P. myriotylum*. The antagonistic and mycoparasitic potential of the *T. asperellum* strains was evaluated *in vitro* through dual culture and interaction tests. Subsequently, the ability of *T. asperellum* to protect cocoyam plants from *P. myriotylum* infection was tested *in vivo*. Our results showed that all four *T. asperellum* strains were antagonistic to *P. myriotylum*, although differences were found among the strains. The growth of *P. myriotylum* was inhibited by more than 60%. Furthermore, the four *T. asperellum* strains were aggressive mycoparasites of *P. myriotylum*. In *in vivo* trials, pretreatment of cocoyam plants with the strains PR10 and PR11 of *T. asperellum* could reduce *P. myriotylum* infection by 50%. Both PR10 and PR11 strains of *T. asperellum* present promising biocontrol potential against *P. myriotylum*.


## 1. Introduction

Due to its high consumption levels and its nutritional and economic values, cocoyam [*Xanthosoma sagittifolium* (L.) Schott.] is an important crop for nearly 13 million people in Cameroon, where its annual production is around 1,668,130 t (FAO, 2009). Root rot, caused by the oomycete *Pythium myriotylum* Dreschler is the major disease that limits growth and productivity of this crop and causes significant economic losses (Nzietchueng, 1983; Zang and Yang, 2000). Various strategies, consisting of chemical, genetic and agricultural methods, can be used to fight cocoyam root rot. Chemical control is most effective, especially when multiple treatments are applied. Yet this method presents a range of negative side effects such as environmental pollution, detrimental health effects for farmers and consumers, and the risk of emergence of resistant pathogen strains. In view of these serious drawbacks, the development of more environmentally friendly control methods, such as biological control using antagonistic microorganisms, can help to complement current strategies for integrated management of the disease. Tambong and Höfte (2001) used the bacteria *Pseudomonas aeruginosa* for biological control of cocoyam root rot. They showed that *P. aeruginosa* significantly reduced root rot in cocoyam grown in the greenhouse, thus indicating that microorganisms can be effective in controlling this disease.

Fungal species belonging to the genus *Trichoderma* are ubiquitous organisms that can be found often on decaying material as well as in plant rhizospheres (Kubicek et al., 2008; Jaklitsch, 2009). These opportunistic and nonpathogenic plant symbionts can very easily be isolated and grow readily on a variety of substrates. Moreover, *Trichoderma* spp. are generally equipped with antagonistic functions based on various mechanisms such as hyperparasitism, antibiosis and competition (Elad, 2000; Vinale et al., 2008). In addition to these properties, the ability of certain *Trichoderma* species to induce plant resistance against some plant pathogens, promote plant growth and improve photosynthetic activity of plants greatly boost these microorganisms' biological arsenal (Samuels, 2006; Shoresh et al., 2010). Several studies have shown the potential of certain *Trichoderma* strains to control *Pythium* species (Benhamou and Chet, 1997; Aerts et al., 2002; Le et al., 2003; John et al., 2010; Muthukumar et al., 2010; Zamanizadeh et al., 2011).

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Very often, an antagonist can be found in the same ecological niche as the pathogen itself (Krauss et al., 1998). In Cameroon, strains of *Trichoderma asperellum* Samuels Lieckf. & Nirenberg have been isolated from soil samples taken from the rhizosphere of cocoyam plants exhibiting symptoms of root rot. Evaluations of the ability of these strains to control cocoa black pod caused by the oomycete *Phytophtora megakarya* have yielded promising results (Tondje et al., 2007; Deberdt et al., 2008). Therefore, the objective of this study was to determine the ability of these four strains of *T. asperellum* to reduce root rot caused by *P. myriotylum*.

## 2. Material and methods

### 2.1. Fungal material

Strain P60 of *P. myriotylum*, isolated in 2005 from the roots of a cocoyam plant presenting symptoms of root rot in Ekona (south-western Cameroon), was used. It was identified morphologically and molecularly characterised by esterase analysis and polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the rDNA (Adiobo 2006). This strain is preserved in the mycological collection of the Biotechnology Laboratory of the Ekona Regional Centre for Agricultural Research (BLERCAR). Four *T. asperellum* strains used in this study, PR10 (GJS 02-65), PR11 (GJS 02-64), PR12 (GJS 02-66) and 659-7 (GJS 02-63)] (Samuels et al. 2010), were from the IRAD's Regional Laboratory for Biological Control and Applied Microbiology. All these strains originated from soil samples of agricultural fields and their antagonistic potential against *P. megakarya* has previously been demonstrated by Tondje et al. (2007). Unless otherwise stated, plates containing *T. asperellum* and/or *P. myriotylum* strains were incubated at $25 \pm 2$ °C under continuous illumination.

### 2.2. In vitro antagonism tests

Confrontation tests between strains of *T. asperellum* and *P. myriotylum* were conducted *in vitro* using a slightly modified protocol described by Benhamou and Chet (1997). The four *T. asperellum* strains and the *P. myriotylum* strain were grown separately on potato dextrose agar (PDA) for five days. Subsequently, agar disks (3 mm Ø) of each *Trichoderma* and *Pythium* strain were punched using a cork borer and then opposed equidistantly at 80 mm from each other, in a Petri dish containing 15 ml of PDA medium. Petri dishes containing only a plug of *T. asperellum* or *P. myriotylum* constituted controls. Five plates were used for each confrontation (*P. myriotylum* – *T. asperellum*) and three replicates for the controls. Petri dishes were incubated for six days. Subsequently, the ability of *T. asperellum* strains to reduce the expansion of *P. myriotylum* colonies was assessed by determining first whether an inhibition zone could be observed. If not, the action of competition and, in particular, the invasion of *P. myriotylum* by *T. asperellum*, was evaluated from the point of contact between the two strains.

The mycoparasitic activity of the *T. asperellum* strains was assessed using the pre-colonised plate method (Krauss et al., 1998; Tondje et al., 2007). Three replicate plates, pre-colonised with *P. myriotylum*, were inoculated with a strip of PDA medium ($1 \times 6$ cm) colonised by one of the four *T. asperellum* isolates. This was done by gently excising a strip of $1 \times 6$ cm containing the mycelium of *T. asperellum* from a fifth-day-old culture. The strip was carefully placed in a Petri dish fully pre-colonised by *P. myriotylum*, with the surface covered with the *T. asperellum* mycelium in contact with the mycelium of *P. myriotylum*. The position of the strip containing the *Trichoderma* inoculum was marked on the bottom of each Petri dish. Inoculated pre-colonised plates were observed daily to check for growth of the inoculated *Trichoderma*. Growth of the *Trichoderma* was measured as described by Krauss et al. (1998). On each sample date, one of the six perpendicular strips was removed using a scalpel, cutting from the distal edge towards the mycoparasite inoculum to avoid cross-infection. The agar strips were then cut into 6 successive 1-cm-pieces so that the presence of the mycoparasite on a particular $1 \times 1$-cm block, which had a known distance from the original inoculum strip, could be related to the growth made across the pre-colonised plate in the time between initial inoculation and sampling. These blocks were placed in a Petri dish containing PDA amended with benomyl at 50 mg $l^{-1}$. The role of benomyl is to inhibit the growth of *T. asperellum*. The block derived directly from beneath the inoculation strip served as positive control. After three days of incubation, the level of mycoparasitism and the progression of mycoparasites were determined by the presence or absence of the growth of *P. myriotylum* indicating its destruction by the *T. asperellum* strains. The whole experiment was repeated twice.

### 2.3. In vivo antagonism tests

Pure conidia of the *T. asperellum* strains used in the *in vivo* tests were obtained through solid-state fermentation as described by Hebbar and Lumsden (1999). *T. asperellum* strains were grown on PDA for five days. Ten agar disks (5 mm Ø), containing the mycelium of each strain of *T. asperellum* were extracted and used to inoculate 100 g of sterile husked boiled rice contained in polyethylene fermentation bags (Hannada et al., 2009). Inoculated bags were then incubated in the laboratory for 10 days. When completely colonised, the substrate was dried under conditioned air and the pure conidia were extracted using a Mycoharvester version V following the manufacturer's protocol (http:/www.dropdata.net/mycoharvester). After drying, 1 g of conidia of each *T. asperellum* strain was introduced into 100 ml of sterile distilled water and the concentration was adjusted to $1 \times 10^6$ conidia $ml^{-1}$ using a haemocytometer.

Five dishes containing PDA were inoculated with *P. myriotylum* strain P60. After five days of incubation, each *P. myriotylum* culture was sequentially mixed with 200 ml of sterile distilled water and ground using a mixer. One ml of this suspension was removed and introduced into test tubes, then mixed using a vortex (Labnet, National Labnet Co., Inc. Woodbridge, NJ, USA) and diluted twice tenfold. One ml of both dilutions was plated on Petri dishes filled with 15 ml of PDA and incubated for 24 h. Afterwards, the number of colonies of *P. myriotylum* on the plates was used to calculate the concentration of colony forming units (cfu) per ml of suspension and to adjust it to a concentration of $12.5 \times 10^2$ cfu $ml^{-1}$.

The substrate used for the *in vivo* tests was provided by the BLERCAR, Ekona, Cameroon. It consisted of black soil (sterilised twice at 121 °C for 15 min) mixed with perlite in a 4:1 proportion and amended with 6 g of $CaMg(CO_3)_2$, 320 mg $Ca(NO_3)_2$ and 220 mg $KH_2PO_4$/kg. The substrate was first incubated at 60 °C for five days in polyethylene bags and then amended with 860 mg of DCM Ecomix fertiliser (7-7-10) per kg of substrate. Subsequently, the inoculum of the pathogen was incorporated into the substrate in the ratio of 1 ml $g^{-1}$ of substrate. One kg of the substrate thus inoculated was transferred into 15-cm diameter plastic pots.

Plants of a white cocoyam variety, highly susceptible to root rot and obtained through micropropagation, (provided by the BERCAR) were used. After six to eight weeks of growing in a greenhouse, these plants were soaked for 24 h in pure conidia suspensions ($10^6$ conidia $ml^{-1}$) of each of the four *T. asperellum* strains. Subsequently, each plant was transplanted into a pot containing 1 kg of the *P. myriotylum*-inoculated substrate. The control treatment consisted of plants soaked in sterile distilled water. Pots were watered every three days with 250 ml of water to maintain substrate moisture. Each treatment consisted of 10 pots and the experiment was performed twice.

The effects of the treatments were assessed 30 days after transplanting by observing and quantifying disease symptoms that appeared on the leaves. The severity of the disease was characterised using the scale of Tambong et al. (1999), which varies from class 1 to class 5 as follows: 1 = no symptoms of the disease on the leaf; 2 = start of yellowing of the leaf; 3 = pronounced and total yellowing of the entire leaf; 4 = start of drying of the leaf; 5 = total desiccation and death of the entire leaf.

### 2.4. Statistical analyses

The percentage of *P. myriotylum* growth inhibition [*I* (%)] by *T. asperellum* was calculated by the formula

$$I(\%) = (1 - C_n/C_0)*100$$

where *C* is the mean diameter of the colonies in presence of the antagonist ($C_n$) or controls ($C_0$).

Mycoparasitism speeds in mm day$^{-1}$ were calculated for each strain by using the slope of the linear regression of the growth curve (Lahlali et al., 2005; Begoude et al., 2007). The root rot severity index (SI) was calculated using the formula

$$\mathrm{SI} = \frac{\sum_{i=1}^{5} C_i N_i}{\sum_{i=1}^{5} N_i}$$

where $C_i$ represents the symptom class and $N_i$ the number of leaves belonging to the symptom class. Using the obtained data, the percentage disease inhibition (PI) was calculated for each *T. asperellum* strain using the following formula: $\mathrm{PI} = [(\mathrm{SI}_c - \mathrm{SI}_t)/\mathrm{SI}_c] \times 100$, where $\mathrm{SI}_c$ is the severity index of the control and $\mathrm{SI}_t$ is the severity index of the plants treated with one of the four *T. asperellum* strains. The data were analysed using the appropriate General Linear Model (GLM) procedure using SAS (Statistical Analysis System; version 9.1). Duncan's multiple means comparison test was used to classify homogeneous groups.

## 3. Results

### 3.1. Effect of T. asperellum on the growth of P. myriotylum in vitro

The four *T. asperellum* strains showed antagonistic activity against *P. myriotylum* in the direct confrontation tests. Antagonism resulted in the cessation of growth of *P. myriotylum* when in contact with *T. asperellum*. No significant difference in the inhibition percentage of mycelial growth of *P. myriotylum* by each of the four *T. asperellum* strains tested was observed. In comparison with the control, the mean percentage inhibition was about 66% on the sixth day. No inhibition zone was observed and at the contact zone (Fig. 1A–D), a dense sporulation followed by an overgrowth of *T. asperellum* on *P. myriotylum* was observed on the sixth day (Fig. 1E–G).

The four *T. asperellum* strains exhibited mycoparasitic activity. The necrotrophic mycoparasitism was confirmed by the absence of growth of *P. myriotylum* in the selective culture medium. Analysis of variance showed that there was a significant difference ($P < 0.05$) between mycoparasitism speeds of the different *T. asperellum* strains. The highest growth rate was recorded with *T. asperellum* strain PR11 ($10.6 \pm 0.10$ mm day$^{-1}$), followed by PR10 ($10.2 \pm 0.04$ mm day$^{-1}$) and PR12 ($10.1 \pm 0.05$ mm day$^{-1}$). The mycoparasitism speed was lowest for 650-7 ($9.8 \pm 0.06$ mm day$^{-1}$).

### 3.2. Effect of the treatment of the plants on the development of root rot

After 30 days of growth in pots, the plantlets had on average five leaves. No significant difference was observed in the total number of cocoyam leaves across the treatments. The ability of *T. asperellum* strains to reduce the severity of root rot caused by *P. myriotylum* was evaluated *in situ*. Table 1 shows, for each *T. asperellum* strain, the average number of leaves per plant, the percentage leaves in each of the five classes, and the disease severity. The percentage of healthy leaves (class 1) was higher for PR10, PR11, PR12 and 659-7 treatments when compared with the control. Conversely, the percentage of diseased leaves (class 5) was highest for the control plants when compared with the plants treated with the biological control agents. As far as the severity index is concerned, a significant difference ($P < 0.05$) was observed between treatments. The PR11 and PR10 *T. asperellum* strains, with mean severity indices of 1.58 and 1.67 respectively, exhibited the highest reduction in the severity of the infection (Table 1). In fact, the strain PR11 and PR10 reduced diseases severity by more than 50%, with the highest inhibition percentage being obtained for the PR11 strain (56.7%). The PR12 and 659-7 *T. asperellum* strains, on the other hand, were less effective, with inhibition percentages of 31.0 and 43.6%, respectively.

## 4. Discussion

The characterisation of *T. asperellum*'s antagonistic potential against *P. myriotylum* is the first step in evaluating the biocontrol capacity of these agents. In this study, we assessed the potential of four *T. asperellum* isolates as biological agents to control *P. myriotylum*, the causative agent of cocoyam root rot disease. We showed that all *T. asperellum* strains were antagonistic to *P. myriotylum* and could be aggressive mycoparasites.

In the *in vitro* trials, the growth of *P. myriotylum* was indeed, inhibited by all four *T. asperellum* strains. Inhibition of *P. myriotylum*'s mycelial growth was on average 66%. Visual analysis of the development of the confrontation showed the inhibition of *P. myriotylum* growth shortly after coming into contact with the *T. asperellum* strains. This cessation of growth was followed by an all-out invasion of the *P. myriotylum* mycelium by those of the *T. asperellum* strains. When interactions between *T. asperellum* strains and *P. myriotylum* were examined, no diffusible substance causing an antibiosis effect was released by any strain of *T. asperellum*, suggesting that the destruction of *Pythium* is due to the mycoparasitic activity. The idea that mycoparasitism is one of the main ways for *Trichoderma* to combat pathogens is supported by previous studies. For example, John et al. (2010) reported the mycoparasitic activity of *Trichoderma viride* on *Pythium arrhenomanes*, a pathogen of soybean. However, they observed that, initially, *P. arrhenomanes* showed a faster growth leading to more than half of the plate being covered within two days. But later, after five days, *T. viride* overgrew *P. arrhenomanes* and destroyed it completely. In another study, Hibar et al. (2005) showed the invasion of the mycelium of *Fusarium oxysporum* f. sp. *Radicis-lycopersici* by *Trichoderma harzianum* six days after direct confrontation. Furthermore, evidence of mycoparasitic activity was demonstrated via the necrotrophic mycoparasitism test. This test showed a direct development of the *T. asperellum* strains to the detriment of *P. myriotylum*, thus corroborating the results obtained by Tondje et al., (2007). However, the growth rates recorded in our study were 2–5 times higher than those obtained by Krauss et al. (1998) when testing the mycoparasitism of the strain MP11 of *Trichoderma* spp. on different pathogens (*Fusarium* spp., *Colletotrichum* spp., *Botryodiplodia theobroma* and *Nigrospora sphaerica*). In the study of Tondje et al. (2007) where the four *Trichoderma* strains have been used to control cocoa black pod disease, caused by a straminopilous *P. megakarya*, the biological principle of their actions was discussed. According to these authors, the mycoparasitic activity of

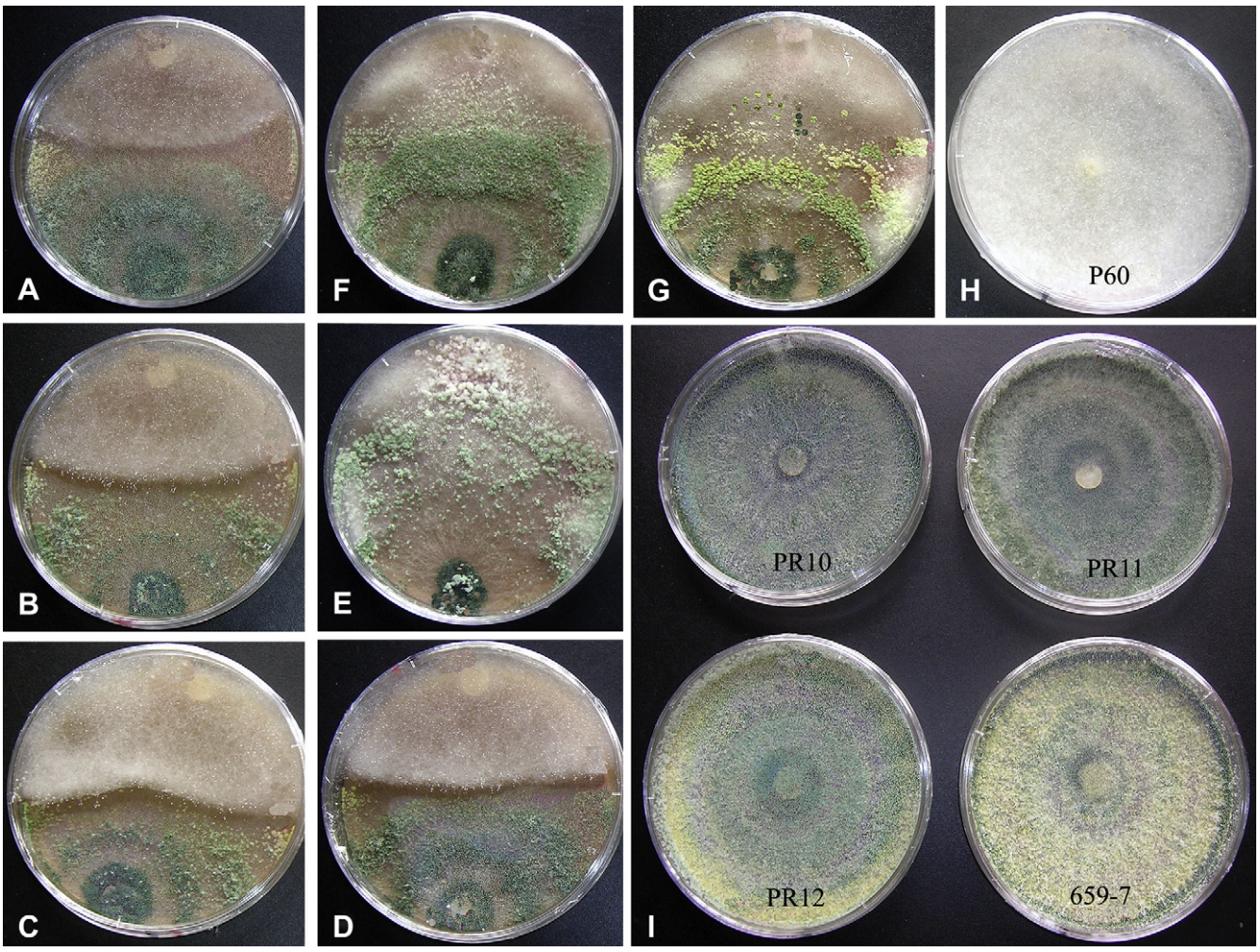


**Fig. 1.** Antagonism of *Trichoderma asperellum* strains against *Pythium myriotylum* following a direct confrontation test: A–D: Contact zone between *P. myriotylum* and strains PR10, PR11, PR12 and 659-7 respectively; E–G: Dense sporulation and overgrowth of *T. asperellum* on *P. myriotylum*; H: growth of the *P. myriotylum* strain; I: growth of *T. asperellum* strains.

the *T. asperellum* strains involves both the direct penetration into the sporocysts and the winding of the pathogen on the hyphae, where a high level of hydrolytic enzymes is produced prior to their destruction.

While often there is lack of correspondence between antagonistic activity *in vitro* and *in vivo* (Holmes et al., 2004; Rosa and Herrera, 2009), our results agree with numerous studies on the beneficial impact of *Trichoderma* spp. *in vivo* (Lahlali and Hijri, 2010; Sant et al., 2010; Affokpon et al., 2011). The *in vivo* tests showed that the four *T. asperellum* strains reduce severity of cocoyam root rot, with the most efficient strains being PR11 and PR10. Indeed, pretreatment of the cocoyam plants with pure cultures of these two *T. asperellum* strains could reduce *P. myriotylum* infection by more than 50%, confirming the *in vitro* results where these strains were found to be the most efficient. Similarly, a study by John et al. (2010) has shown that the application of *T. viride* to a substrate previously inoculated with propagules of *P. arrhenomanes*, causative agent of soybean root rot, led to a 90–100% improvement in the soybean germination rate. These same authors have also shown that chlorophyll size and pigments, and the growth – and hence the yield – were improved when the substrate was treated with *T. viride* propagules.

**Table 1**
Effects of treatments of plants with *Trichoderma asperellum* strains on cocoyam root rot.

| Treatments | NL[a] | Percentage of leaves per class[b] | | | | | SI[c] |
|---|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | C5 | |
| PR10 | 5.30a | 45 | 32 | 18 | 5 | 0 | 1.67d |
| PR11 | 4.80a | 50 | 44 | 6 | 0 | 0 | 1.58d |
| PR12 | 5.20a | 32 | 28 | 22 | 15 | 3 | 2.52c |
| 659–7 | 5.00a | 36 | 30 | 26 | 8 | 0 | 2.06b |
| Control | 5.20a | 7 | 13 | 12 | 27 | 42 | 3.65a |

*Note*: Values in column with different letters are significantly different ($P < 0.05$) according to the Duncan's multiple means comparison test.

[a] NL = Average number of leaves per plant.

[b] SI = Severity Index.

[c] Data presented the percentage of leaves per classes: C1 = leaves with no disease symptoms; C2 = leaves with starting yellowing symptoms; C3 = leaves with pronounced and total yellowing symptoms; C4 = leaves showing a beginning of drying symptoms; and C5 = leaves totally desiccated and dead.

In conclusion, this study showed the antagonistic effect of *T. asperellum* against *P. myriotylum*, causative agent for cocoyam root rot. The confrontation tests between *T. asperellum* and *P. myriotylum* showed an inhibition of the mycelial growth of the pathogen. The mycoparasitism test showed a direct development of *T. asperellum* on the *P. myriotylum* mycelium. Soaking the plants in pure conidial suspensions of certain *T. asperellum* strains led to the reduction in the incidence of root rot by over 50%. These results suggest that *T. asperellum* strains, such as PR11 and PR10, have potential for incorporation into IPM schemes for the control of

cocoyam root rot disease. However, it will be necessary to undertake field trials in order to determine the ability of these strains to protect cocoyam plants against the root rot disease under natural conditions.

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