Antagonism of *Trichoderma asperellum* against *Phytophthora megakarya* and its potential to promote cacao growth and induce biochemical defence

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This work aimed to assess the antagonism effects of four different *Trichoderma asperellum* isolates against *Phytophthora megakarya* and their ability to enhance cacao growth and biochemical defence. Results showed that in paired culture, all the isolates of *T. asperellum* used were antagonistic to *P. megakarya* by means of mycoparasitism. In pot experiments, leaf number, plant height, shoots and root dry matter were significantly increased by *T. asperellum*. Similarly, chlorophyll rate, P uptake and acid phosphatase activities were also increased. These antagonists reduced significantly the effects of *P. megakarya* in the leaves of cacao plants. Amino acid and phenolic components content increased in either healthy or infected leaves from cacao plants inoculated with *T. asperellum*. There was negative correlation between both phenolic compounds and disease severity and amino acids and disease severity. This suggests that these compounds are involved in disease resistance. In fact, the induction of specific amino acids such as alanine, glutamic acid and methionine may play an important role in the adaptation of cacao plant to *P. megakarya* infection. These findings demonstrated that *Trichoderma asperellum* (PR10, PR11, PR12 and PR659-7) could be used to improve the development of cacao plants and protect the plant against *Phytophthora megakarya*.

Introduction

*Theobroma cacao* L., commonly known as cacao, is an important economic crop in numerous developing countries. Cameroon is the fifth largest world cacao producer and its production of merchantable cacao in 2014–2015 was estimated at around 232 thousand tons (ICCO 2016). In this country, cacao is an essential source of revenue for many people. Cacao beans are rich in sugar, unsaturated fatty acids and it is the raw material for industrial chocolate.

In Cameroon, black pod, the most damaging disease of cacao, is mainly due to *Phytophthora megakarya* (Holmes et al. 2003). Without crop protection, yield losses could reach from 80% 100% (Ndoumbe-Nkeng et al. 2004; Guest 2007). The main approach for the control of *P. megakarya* is the use of systemic chemical pesticides like metalaxyl. However, this pesticide may be harmful to the environment and its repeated use could result in resistance in the pathogen population and its toxic residue could be accumulated in the pod (Bateman 2004).

Bio-control has been proposed a lot earlier and has actually been used for pest and disease control (among others) for centuries. Several biological agents have been studied, including strains belonging to fungal genera. Among them, *Trichoderma* spp. are the most frequently isolated species from plant rhizosphere ecosystems (Harman et al. 2004). *Trichoderma* spp. are potential biological control agents and their modes of action include many mechanisms such as competition, antibiosis, mycoparasitism and induction of plant defences (Shoresh et al. 2010). Recent studies have reported that *Trichoderma* spp. could promote plant growth and stimulate the synthesis of compounds such as phenolic compounds, proteins and amino acids (Gravel...
et al. 2007; Shoresh et al. 2010). These compounds may inhibit directly fungal development, or may be implicated in the metabolic pathways associated with diseases resistance (Calvo et al. 2002).

This investigation was carried out to assess the antagonism effects of four *Trichoderma asperellum* isolates against *P. megakarya* and its ability to enhance cacao growth and improve induced biochemical defence mechanisms.

**Materials and methods**

**Microorganisms’ origins**

*P. megakarya* (strain PM5) used in this study was obtained from infected cacao pod (Yaounde-Cameroon) (Tchameni 2014). The zoospore suspension was obtained according to the method used by Sameza et al. (2014). Zoospore concentration was adjusted to $10^6$ conidia ml$^{-1}$ using a hemocytometer for spore counts.

Four isolates of *Trichoderma asperellum* (PR10, PR11, PR12 and PR659-7) used in this study came from the Regional Laboratory of Applied Microbiology and Biological Control of Institute of Agricultural Research for development (IRAD) of Cameroon (Tondje et al. 2007). Each isolate was grown on potatoes dextrose agar medium for 5 days for conidia production. The spore suspension was obtained by washing the surface of the petri dish with fungal colonies with 5 ml of sterile distilled water. The spore suspension was filtered through sterile cheese cloth and the concentration adjusted to $10^6$ conidia ml$^{-1}$ using a hemocytometer for spore counts.

**Seedling production**

Cacao seeds selected came from 6-month pods of cacao tree (SNK213 cultivar), which are susceptible to black pod disease with 62.0% of infection successful. These hand-pollinated pods came from cacao trees in an experimental field (IRAD Nkolbisson, Cameroon). Seeds with similar size and weight were sown in a plastic bag (3 l) half-filled with sterilised soil and incubated for 14 days at room temperature for pre-germination. The growth medium consisted of a mixture of soil (known as Rhodickanduidlut soil) and sand (3:1 v/v). This mixture was autoclaved three times at 24-h intervals for 1 h at 121°C and transferred into 3-l plastic pots (3 kg/pot).

**Plant growth**

A pre-germinated cacao seedling was seeded into each pot and 5 ml of each strain of *T. asperellum* conidial suspension was inoculated around the seedlings, while control pots received 5 ml of sterilised distilled water. Each treatment consisted of five replicates. Plants received 200 ml distilled water every two days and were grown for 18 weeks.

**In vitro antagonism tests**

It was done by dual culture to verify that the isolates of *Trichoderma* had not lost any of their potential over time as previously described by Tondje et al. (2007).

**Plant sampling and analysis**

Eighteen weeks after planting, leaf number and plant height of each plant were recorded. The chlorophyll rate was evaluated *in situ* using chlorophyll meter apparatus. Plants were harvested, roots and shoots dry matter was determined separately after drying at 70°C for 72 h. Dried shoots were mineralised in concentrated sulphuric acid at 480°C for 4 h and P content determined using the method of Okalebo et al. (1993). Extract for acid phosphatase activity in fresh roots was obtained as described by Tarafdar and Marschner (1994). Here, root samples (1 g) were ground with a mortar and pestle in 5 ml of 0.4 M sodium acetate buffer (pH = 5). The extracts were centrifuged at 5000 rpm for 10 min at 4°C, and the specific acid phosphatase activity was evaluated in the supernatant according to the method used by Tchameni et al. (2011).

**Leaf inoculation and disease severity**

The resistance degree of cacao plant was evaluated by infecting detached leaves with a zoospore suspension of *P. megakarya*. Five leaves were used for each treatment and the experiment was designed as described by Nyasse et al. (1995). For inoculation, 10 µl of zoospore suspension ($10^6$/ml) were deposited on the underside of five different locations of each leaf. The control received 10 µl of sterile distilled water. Leaves were incubated at 25 ± 2°C in the dark for 5 days and symptoms were assessed using the lesion scoring scale (Nyasse et al. 1995) and the experiment was repeated.
twice. The level of resistance was expressed as follows: very susceptible (VS: 3.5 < score ≤ 5), susceptible (S: 2.5 < score ≤ 3.5), slightly resistant (SR: 2 < score ≤ 2.5), resistant (R: 1 < score ≤ 2) and very resistant (VR: 0 < score ≤ 1) (Paulin et al. 2008).

**Evaluation of soluble phenolic compound content and amino acid identification**

Sections of leaves 1 cm beyond necrosis or the infection point (when no symptoms occurred) were used for analysis. Extraction and determination of phenolic content was carried out according to the method used by Tchameni et al. (2011). Extraction of total soluble amino acids was performed according to the method of Singh et al. (1990). The total soluble amino acid content and their identification were determined as described by Omokolo et al. (2002) after separating them by thin layer chromatography.

**Statistical analysis**

Analysis of variance using SAS (Statistical Analysis System software version 9.1) was used to analyse data. The degree of significance between means was determined at 5% using the Duncan test. Pearson correlation was used to evaluate the relationship between pairs of variables.

**Results**

**In vitro antagonistic activities of T. asperellum against P. megakarya**

All the isolates of *T. asperellum* used were antagonistic to *P. megakarya* by means of mycoparasitism. The mean inhibition percentage of *P. megakarya* growth by *Trichoderma* was 75%, 80%, 70% and 66%, respectively, for PR10, PR11, PR12 and PR659-7. Moreover, attempts to recover microorganisms from the intermingling region lead to the growth of each strain of *T. asperellum* only, showing that *P. megakarya* did not survive in the presence of *Trichoderma*.

**Effects of T. asperellum on growth parameters of cacao seedlings**

Eighteen weeks after planting, plant height, leaf number, root and shoot dry matter were variably affected (Table 1). PR11 and PR12 significantly increased leaf number and plant height, compared to the control. Plant dry matter increased in the presence of all the tested isolates of *T. asperellum*. The increase in shoot dry matter was 62.5%, 70%, 60% and 62%, respectively, by PR10, PR11, PR12 and PR659-7. These isolates also increased root dry matter by 72.72%, 136.36%, 118.18% and 89.10%.

**Effects of T. asperellum on chlorophyll, phosphorus content and acid phosphatase activity of cacao seedlings**

All the isolates of *T. asperellum* variably affected the biochemical parameters of cacao seedlings after 18 weeks of planting (Table 2). Plants inoculated with PR11 and PR12 had a significantly high chlorophyll rate compared to the control. Moreover, all the isolates of *T. asperellum* significantly increased both the phosphorus content and the acid phosphatase activities. Phosphorus content in the control was 5.75 µg/g of shoot dry matter, while in plants treated with PR10, PR11, PR12 and PR659-7, it increased to 10.15 µg/g (a 1.76-fold increase), 12.4 µg/g (a 2.15-fold increase), 15.20 µg/g (a 2.64-fold increase) and 11.15 µg/g (a 1.94-fold increase), respectively. The increase of acid phosphatase activity was 2.0 fold, 2.33 fold, 1.5 fold and 1.33 fold for PR10, PR11, PR12 and PR659-7 treatment, respectively.

**Effect of T. asperellum on P. megakarya leaf disease severity**

Results show that when the plants were inoculated with the different isolates of *T. asperellum*, disease severity was significantly (*P* ≤ 0.05) reduced (Figure 1). On the control plants, the disease rate

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**Table 1.** Effect of four strains of *T. asperellum* (PR10, PR11, PR12 and PR659-7) inoculation on growth response of cocoa plants 18 weeks after planting.

| Treatments | Height (cm) | Leaf number (per plant) | Shoot dry matter (g/plant) | Root dry matter (g/plant) |
|------------|-------------|-------------------------|---------------------------|--------------------------|
| Control    | 26.0<sup>a</sup> | 14.0<sup>a</sup> | 2.0<sup>a</sup> | 1.10<sup>a</sup> |
| PR10       | 27.2<sup>b</sup> | 15.0<sup>b</sup> | 3.25<sup>b</sup> | 1.90<sup>b</sup> |
| PR11       | 37.9<sup>b</sup> | 22.0<sup>b</sup> | 3.40<sup>b</sup> | 2.6<sup>b</sup> |
| PR12       | 36.43<sup>b</sup> | 21.0<sup>b</sup> | 3.20<sup>b</sup> | 2.4<sup>b</sup> |
| PR659-7    | 25.5<sup>b</sup> | 15.0<sup>b</sup> | 3.24<sup>b</sup> | 2.08<sup>b</sup> |

According to the Duncan test, means with the same letter within the same column are not significantly different at *P* < 0.05. Each treatment had five replicates and was repeated twice.
was 3.5 (i.e. susceptible or very susceptible), while in leaves from plants treated with *Trichoderma*, it was 1.5 (i.e. resistant), for PR10 and PR12; 1.0 (i.e. resistant or very resistant) for PR11 and 2 (i.e. resistant or slightly resistant) for PR659-7.

**Total soluble phenolic compound content of leaf**

The data presented in Figure 2 show that the plants inoculated with the antagonist exhibited significant high levels of phenolic compounds in the leaves when compared to the control. Total phenol content in the control was 2.33 mg/g dry weight, whereas in the plant leaves treated exclusively with isolates of *T. asperellum*, the total soluble phenolic compound was 3.71 mg/g dry weight (59.22% increase), 4.32 mg/g dry weight (85.40% increase), 3.3 mg/g dry weight (1.28-fold increase) and 2.64 mg/g dry weight (1.61-fold increase) for PR10, 6.75 mg/g dry weights (1.56-fold increase) for PR11, 4.32 mg/g dry weight (1.28-fold increase) and 3.3 mg/g dry weight (1.25-fold increase) for PR659-7.

Total phenolic component accumulation was significantly induced by *P. megakarya*, 5 days after infection. In the control leaves, the amount of soluble phenolic compound was higher, at 3.35 mg/g dry weight (1.44-fold increases). From plant leaves inoculated with isolates of *T. asperellum*, the total soluble phenolic compound was 5.98 mg/g dry weight (1.61-fold increase) for PR10, 6.75 mg/g dry weights (1.56-fold increase) for PR11, 4.32 mg/g dry weight (1.28-fold increase) and 3.3 mg/g dry weight (1.25-fold increase) for PR659-7. Negative correlations (*P* = 0.002; *r*² = −0.86) were found between the level of total soluble phenol content and disease index.

**Total soluble amino acid content of leaf**

Results showed significant differences in the amino acid content of leaves between the control and plants inoculated with different isolates of *T. asperellum* (Figure 3). The amino acid content in the control was 14.05 µg/g of fresh weight, whereas the plants treated with isolates of *T. asperellum* had an amino acid content of 28.11 µg/g fresh dry weight (41.63% increase) and 2.64 mg/g dry weight (13.30% increase) were noted, respectively, for PR10, PR11, PR12 and PR659-7.

### Table 2. Effect of four strains of *T. asperellum* (PR10, PR11, PR12 and PR659-7) inoculation on chlorophyll, P and acid phosphatases on cocoa plants 18 weeks after planting.

| Treatments | Chlorophyll (%) | Phosphorus (µg/g of plant) | Acid phosphatase (µmol/min/mg of protein) |
|------------|-----------------|-----------------------------|------------------------------------------|
| Control    | 26.0 ± a        | 5.75 ± a                    | 0.03 ± a                                 |
| PR10       | 27.0 ± a        | 10.15 ± b                   | 0.06 ± c                                 |
| PR11       | 37.5 ± b        | 12.4 ± b                    | 0.07 ± c                                 |
| PR12       | 29.1 ± b        | 15.20 ± b                   | 0.045 ± c                                |
| PR659-7    | 26.1 ± b        | 11.15 ± b                   | 0.04 ± c                                 |

According to Duncan test, means with the same letter within the same column are not significantly different at *P* < 0.05. Each treatment is made of five replicates and was repeated twofold.

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**Figure 1.** Effect of four strains of *T. asperellum* (PR10, PR11, PR12 and PR659-7) inoculation on *Phytophthora megakarya* disease severity on the leaves of cocoa plants. According to the Duncan test, means with the same letter are not significantly different at *P* < 0.05. Each treatment had five replicates and the experiment was repeated twice.
weight (2.0-fold increase), 25.56 µg/g fresh weight (1.82-fold increase) and 19.17 µg/g fresh weight (1.36-fold increase), respectively, for PR10, PR11, PR12 and PR659-7. Amino acid accumulation was significantly induced against pathogen infection in leaves from the control plant and from *T.*

Figure 2. Effect of four strains of *Trichoderma asperellum* and *Phytophthora megakarya* infection on soluble phenol in leaves of cocoa seedlings. According to the Duncan test, means with the same letter are not significantly different at *P* < 0.05. Each treatment had five replicates and the experiment was repeated twice.

Figure 3. Effect of four strains of *Trichoderma asperellum* and *Phytophthora megakarya* infection on soluble amino acids in leaves of cocoa seedlings. According to the Duncan test, means with the same letter are not significantly different at *P* < 0.05. Each treatment is made of five replicates and was repeated twofold.
asperellum}-treated plants. There was a significant and negative correlation ($P = 0.0002$; $r^2 = -0.65$) between amount of amino acids and disease severity.

**Amino acid identification**

From the results (Table 3) we noticed that compared to the control, the amino acid profile was significantly modified after cacao plants were inoculated with isolates of *T. asperellum* and after infection with *P. megakarya*. In the control (non-infected leaves), four amino acids were identified, namely tyrosine, serine, proline and leucine. In leaves inoculated only with *T. asperellum*, the amino acid profile was modified as follows: alanine, glutamic acid and methionine (except PR 10) were present while tyrosine disappeared.

After infection of the cacao leaves with the pathogen, the amino acid profile was as follows: the presence of alanine, glutamic acid and methionine and the disappearance of leucine were noted in the leaves of the control plant and the plants treated with all isolates of *T. asperellum*.

**Discussion**

The results from this investigation showed that the four isolates of *T. asperellum* were antagonistic to *P. megakarya* and could be aggressive mycoparasites. Under the same conditions, all the tested antagonist isolates grew significantly faster than *P. megakarya*. Tondje et al. (2007) obtained similar results. Previous works proved that *Trichoderma* spp. is a potential bio-control agent of several plant pathogens including *P. megakarya* (Vinale et al. 2009; Reithner et al. 2014; Thakkar & Saraf 2015). *Trichoderma* takes advantage of its rapid growth to compete for the space and nutrients with plant pathogenic fungi (Simon & Sivasithaparam 1988). The mycoparasitism test showed a direct development of each strain of *T. asperellum* to the disadvantage of *P. megakarya*. This parasitism was necrotrophic since *P. megakarya* could not be recovered from hypha zones. The mycoparasitic activity may be due to the direct penetration of *Trichoderma* into the sporocysts or its coiling around the pathogen hyphae, to antibiosis and the leakage of wall-degrading enzymes (Benitez et al. 2004; Thakkar & Saraf 2015).

Pot experiments showed that *T. asperellum* significantly increased the growth parameters of cacao seedlings. This finding is in agreement with Martínez-Medina et al. (2011), Hermosa et al. (2012) and Viterbo et al. (2012), who found that *Trichoderma* significantly increased root and shoot dry weight in tomato and melon, respectively. The chlorophyll rate and phosphorus content in the dry shoot of cacao plant treated with *T. asperellum* were significantly higher than control plants. Yedidia et al. (2001) showed that the treatment of cucumber seedlings with *Trichoderma* resulted in a significant increase in the concentration of many compounds including chlorophyll and phosphorus. These results may be explained in part by the findings of Altmare et al. (1999); Viterbo et al. (2010) and Martínez-Medina et al. (2011) who reported that *Trichoderma* increased nutrient availability in the soil, which may lead to increased nutrient uptake and accumulation in plants. Rubio et al. (2014) suggested that *Trichoderma* secrete root-
growth-stimulating substances. In this present study, inoculation with isolates of *T. asperellum* stimulated root acid phosphatase activity, enzyme involved in the phosphorus uptake by plants in soil. Cacao tree is a perennial plant. Evaluation of their fruit resistance against black pod has taken several years. To assess their tolerance against *P. megakarya*, early tests were developed on detached leaves from young plants (Nyasse et al. 2002; Paulin et al. 2008). Pathogenicity tests showed that leaf disease symptoms were significantly reduced in plants treated with *T. asperellum* isolates. Many studies have reported that *Trichoderma* spp. could control some plant pathogens including members of the genus *Phytophthora* (Hanada et al. 2008; Vinale et al. 2008; Carvalho et al. 2015). Deberdt et al. (2008) actually argue that the frequent spraying of pods by *T. asperellum* actively protect the pods from *P. megakarya*. Results from these studies suggested that the mechanism of disease suppression could be the induction of systemic resistance since there was no direct contact between *P. megakarya* and *T. asperellum* within the plant. The accumulation of defence compounds such as amino acids, phenols and pathogenesis-related proteins is among the most common known mechanism induced in plants after infection with inducing agents (Vinale et al. 2008). This occurrence was established in our study by the significant high accumulation of amino acids and phenols in the leaves of cacao seedlings following *T. asperellum* inoculation in comparison to the control plants. The high level of phenols and amino acids in cacao leaves infected with *P. megakarya* suggests that these metabolites are involved in disease resistance. Similar observations have been made by Omokolo et al. (2002) and Djocgoue et al. (2011), who showed that the amino acid and phenolic content are increased in cacao leaves after infection. Moreover, the induction of amino acids and phenolic compounds in cacao plants could be a potential mechanism for their adaptation to *P. megakarya* tolerance. The profile of amino acids after infection revealed a disappearance of tyrosine. This amino acid is well known as a precursor for the synthesis of phenolic compounds, pathogenesis-related proteins, which are molecules involved in plant defence mechanism (Palma et al. 2002; Van Loon et al. 2006). The main amino acids appearing after *T. asperellum* inoculation and leaf infection were alanine, glutamic acid and methionine. Appearance and disappearance of some amino acids after infection reveal that their metabolic pathways are oriented. This finding is in agreement with many studies that demonstrated that some specific amino acids such as cysteine, arginine and threonine are synthesised in plants after biotic stress (Nemec 1995; Omokolo et al. 2002; Tchameni 2014).

**Conclusion**

Our study showed the antagonistic effects of four isolates of *T. asperellum* against *P. megakarya*. These isolates enhanced cacao growth and reduced disease incidence and also induced the accumulation of phenolic compounds and some amino acids, which are considered to be involved in plant defence mechanisms. The ability of *T. asperellum* to boost plant growth and improve plant defence varies from one isolate to another. However, identification of induced phenolic compounds in cacao leaves and metabolites from *T. asperellum* is needed to fully understand the process.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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