MYCOPARASITIC CAPABILITY OF TRICHODERMA ASPERELLUM AND ITS METABOLITE

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ABSTRACT
The antimicrobial ability of Trichoderma asperellum metabolite against Penicillium oxalicum, Botryodiplodia theobromae and Aspergillus niger was examined. The antifungal ability of the Trichoderma in dual culture with each of the three fungi was also examined. All fungi were obtained from the Germplasm Health Unit, International Institute of Tropical Agriculture, Ibadan, Nigeria. All fungi were sub cultured onto Petri plates of acidified Potato Dextrose Agar (APDA) and incubated at room temperature. Pure cultures of T. asperellum and each of the three fungi was transferred separately onto opposite ends of 9 cm Petri plates of APDA using sterile 2 mm diameter cock borer. Inoculation of separate fungus only served as control. All Petri plates were incubated at 25 ± 2°C for 7 days. Percentage inhibition of each fungus by T. asperellum was later measured. Trichoderma asperellum metabolite was extracted using n-hexane, n-butanol and Potato Dextrose Broth. It was further purified by passing it through 0.22 μm Millipore filter. Six millimetre disc of each fungus was later cultured on separate PDA plates containing 1ml of 10%, 25%, 50% and 100% T. asperellum metabolite. Inoculated PDA plates with no metabolite served as control. All experiments were done in triplicates and incubation was done at 25± 2°C for 7 days. Radial growths of the fungi were recorded at 24 hour interval. The data collected was analysed at IITA using SAS (version 9.3). Growth inhibitions of A. niger, P. oxalicum and B. theobromae in dual culture differed significantly \((P<0.05)\) from the control. Growth inhibitions of the three fungi by all concentrations of the metabolite examined also differed significantly \((P\leq0.05)\) from control. Inhibitions of the three fungi increased significantly \((P\leq0.05)\) with increase in metabolite concentration. Total growth inhibitions of the three fungi were obtained by 100% metabolite concentration \((P\leq0.05)\). Growth inhibition of P. oxalicum was significantly higher than that of A. niger and B. theobromae. Growth inhibition of A. niger was also significantly higher than that of B. theobromae \((P<0.05)\). T. asperellum could thus be said to possess a consistent mycoparasitic potential of wide range in nature. Its potential might be said to depend partly or wholly on its ability to produce antifungal compound(s) and to compete for space and nutrients. Trichoderma asperellum metabolite may be said to possess promising mycoparasitic potential, even at lower concentrations. A linear relationship may also be said to exist between the metabolite’s concentration and growth inhibition of fungi.
Keywords: Growth inhibition, metabolite, T. asperellum, P. oxalicum, A. niger, B. theobromae.

INTRODUCTION

Several species of fungi have been documented as pre and postharvest pathogens of different crops at different times. Such include amongst others the Penicillium, Aspergillus, Fusarium, Colletotrichum, and Botryodiplodia. Species of Aspergillus, Botryodiplodia Penicillium and Fusarium which are reported to be among the major cause of rots in crops both in the field and in storage causing great loss in agricultural production. They are known to have a wide host range, especially among young tissues and seeds of different crops in the field and during storage (Sivan et al., 1984; Agrios, 2005). They are also among the most important mycotoxin producers which are known to colonize and infect grains in storage, thereby causing reduction in feed and market value as well as decreased viability (Wiese, 1977, Schurteff, 1980; Grolleaud, 2002; Adegbite et al., 2005; Dean et al., 2012). Their presence leads to deterioration and spoilage of foodstuffs, whilst exposure to their spores frequently results in allergic reactions, often of a serious nature. Nielsen et al. (2009) reported that A. niger for instance infects crops such as maize, peanuts, grapes, and grape products, coffee, tea, beans, spices. It causes a common postharvest disease called black mould on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It has been reported that the infection of onion seedlings by A. niger can become systemic, manifesting only when conditions are conducive in which the black conidia can be observed between the scales of the bulb. Some strains of A. niger have been reported to produce potent mycotoxins called ochratoxins (Abarca et al., 1994). Frisvad et al. (2007) also reported the production of Fumonisins B2 by A. niger. Some researchers have a contrary opinion, claiming this report is based upon misidentification of the fungal species. Samson et al. (2001) however, gave evidence that suggested some A. niger strains do produce ochratoxin A. The pathogenicity of A. niger has also been reported by CIMMYT (2004), Bansod and Rai (2008), Somda et al. (2008), Person et al. (2010), as well as Datt and Sharma (2014).

Penicillium oxalicum has also been recognized as an important pathogen of certain crops (CIMMYT, 2004). The species has been reported to be the cause of bronchopulmonary penicilliosis (Rippon, 1982). Botryodiplodia theobromae is as well a documented pathogen of several crops. A typical example is the postharvest black kernel rot disease it causes in maize (CIMMYT, 2004).

Trichoderma species have been documented to be very good mycoparasites producing a wide range of antibiotic substances which make them efficient mycoparasites
They are known to produce lytic and proteolytic enzymes, diffusible or volatile metabolites and other secondary metabolites as active measures against their targets (Benitez et al., 2004, Kredics et al., 2005, Dababat et al., 2006, Harman, 2006, Kyalo et al., 2007, Suarez et al., 2007, Goswami et al., 2008, Reino et al., 2008, Chen et al., 2009, Ruocco et al., 2009, Stoppacher et al., 2010, Tisch and Schmoll, 2010, Tran, 2010, Mukherjee et al., 2012). Many *Trichoderma* species have been reported as effective biological control agents against a wide range of crop diseases (Dubey et al., 2007 and Abo-Elyoussr et al., 2014). *Trichoderma harzianum*/*T. polysporum* for instance have been used for the control of several soil borne plant and seed pathogenic fungi as well as the control of wood decays (Agrios, 2005). *Trichoderma asperellum* has also been reported to be a good antagonist that inhibits the growth and development of other fungi (Benitez et al., 2004).

The health hazards posed by chemical control measures for pathogens underscores the need for continuous search for alternative disease control measures such as biological control agents and their antifungal metabolites (Intana et al., 2008, Seema and Devaki, 2012). This study was thus aimed at examining the growth inhibitory potential of different concentrations of *T. asperellum* metabolite on *A. niger*, *P. oxalicum* and *B. theobromae*.

**MATERIALS AND METHODS**

**Sample collection and maintenance** The potential antagonist *T. asperellum* and the three other fungi, namely, *Aspergillus niger*, *Penicillium oxalicum* and *Botryodiplodia theobromae* were obtained from the Germplasm Health Unit (GHU) of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. All fungi were sub cultured onto several Petri plates of acidified Potato Dextrose Agar (APDA) and incubated at room temperature. Stock cultures were later prepared and stored at 28 ± 2.0 for 5 days. The experiment was carried out both at the Germplasm Health Unit of IITA, Nigeria and Africa Rice Centre, Cotonou, Benin.

**Assaying antagonistic potential of T. asperellum in dual culture**

The antagonistic activity of *T. asperellum* against *P. oxalicum*, *B. theobromae* and *A. niger* was examined in dual culture using the method of Khare et al.(2010). A sterile 2
mm diameter cock borer was used to transfer pure cultures of *T. asperellum* and *A. niger* onto opposite ends of 9 cm Petri plates of APDA. This was also done for *T. asperellum* and *P. oxalicum* as well as *T. asperellum* and *B. theobromae*. There were three replications for each treatment. Inoculation of separate fungus only served as control. All Petri plates were incubated at 25 ± 2°C. Percentage inhibition of each fungus by *T. asperellum* was measured using the formula:

\[
\text{Percentage Inhibition} = \frac{(R_1 - R_2)}{R_1} \times 100; \quad \text{where: } R_1 \text{ is Radial growth of the pathogen in control plate, and } R_2 \text{ is Radial growth of the pathogen in the test plate.}
\]

**Extraction and antifungal assay of *T. asperellum* metabolite**

The modified methods of Seema and Devaki (2012) and Jeyaseelan *et al.* (2012) were used for the extraction. Potato Dextrose Broth (PDB) and two solvents (n-hexane and n-butanol) were used for the extraction. Mycelia discs from 7 day-old cultures of *T. asperellum* was inoculated into separate 250 ml flask containing 100 ml 1/4 strength Potato Dextrose Broth (PDB), 50% n-hexane and 50% n-butanol. The flasks were agitated on a rotary shaker at 160 rpm for 5 days at room temperature. Spores and mycelia of the fungi were removed from broth culture by centrifuging at 9000 rpm for 20 mins. The supernatant was filtered through Whatmann no1 filter paper, and it was further purified by passing it through 0.22 mm Millipore filter (Jeyaseelan *et al.*, 2012). Four different concentrations (10%, 25%, 50% and 100%) of the metabolite were prepared. This was done by diluting absolute extract of the metabolite (100% concentration) with sterile distilled water in the ratio 1:9 (10%), 1:3 (25%) and 1:1 (50%). One millilitre of each concentration was then transferred into separate sterile Petri plates. Sterilized molten PDA (20 ml) was later dispensed into each Petri plate. All experiments were done in triplicates and all plates were left to gel before 6 mm disc of each pathogen was inoculated separately onto the centre of the Petri plates. Inoculated plates with no metabolite served as control. Incubation of all plates was done at 25± 2°C for 7 days.

**Data collection and statistical analysis**

The Petri plates were examined daily at 24 hour interval and the radial growth of the pathogens was recorded daily. Percentage inhibition of pathogen was calculated using the formula above. The data collected was analyzed at IITA using SAS (version 9.3). Means separation was done using the Duncan Multiple Range Test (DMRT).
RESULTS

Inhibition potential of *T. asperellum* in dual culture Table 1 shows the growth inhibitory activity of *T. asperellum* against the three fungi. The *Trichoderma* exhibited appreciable inhibitory activity against the three fungi in culture compared to their control. Growth inhibition of *T. asperellum* on *P. oxalicum* was higher than that on *A. niger* and *B. theobromae*. Better inhibition on *A. niger* was also observed compared to that on *B. theobromae*. Generally, higher percentage inhibitions on the three plant pathogenic fungi were observed with longer culture duration (Table 1).

**Table 1: Inhibitory effect of *T. asperellum* on radial growth (cm) of three different fungi**

<table>
<thead>
<tr>
<th>Day</th>
<th>Botryodiplodia theobromae</th>
<th>Aspergillus niger</th>
<th>Penicillium oxalicum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trt</td>
<td>Control</td>
<td>% inhibition</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>5.2</td>
<td>30.8</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>7.35</td>
<td>44.2</td>
</tr>
<tr>
<td>4</td>
<td>3.9</td>
<td>7.8</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>3.6</td>
<td>8.5</td>
<td>57.6</td>
</tr>
<tr>
<td>6</td>
<td>3.6</td>
<td>8.5</td>
<td>57.6</td>
</tr>
<tr>
<td>7</td>
<td>3.6</td>
<td>8.5</td>
<td>57.6</td>
</tr>
</tbody>
</table>

Trt: Radial growth of fungus (cm) in culture with *T. asperellum*

Each value is a mean value of 3 replicates.
**Figure 2:** Total growth inhibition of *Aspergillus niger* by 100% *Trichoderma asperellum* metabolite

**Table 2:** Effectiveness of different concentration levels of *T. asperellum* metabolite against growths of three fungi

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>A. niger</th>
<th>B. theobromae</th>
<th>P. oxalicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Mean % Inhibition</td>
<td>Mean % Inhibition</td>
<td>Mean % Inhibition</td>
</tr>
<tr>
<td>N-Butanol (100%)</td>
<td>100a</td>
<td>100a</td>
<td>100a</td>
</tr>
<tr>
<td>N-Butanol (50%)</td>
<td>76.58b</td>
<td>89.99b</td>
<td>0.10a</td>
</tr>
<tr>
<td>N-Butanol (25%)</td>
<td>56.08c</td>
<td>72.74c</td>
<td>2.57b</td>
</tr>
<tr>
<td>N-Butanol (10%)</td>
<td>42.68d</td>
<td>69.38c</td>
<td>7.35c</td>
</tr>
<tr>
<td>N-Hexane (100%)</td>
<td>31.05f</td>
<td>5.06d</td>
<td>6.92d</td>
</tr>
<tr>
<td>N-Hexane (50%)</td>
<td>14.04g</td>
<td>2.21f</td>
<td>4.78f</td>
</tr>
<tr>
<td>N-Hexane (25%)</td>
<td>10.14h</td>
<td>3.94g</td>
<td>3.92f</td>
</tr>
<tr>
<td>N-Hexane (10%)</td>
<td>2.28i</td>
<td>3.50g</td>
<td>5.71g</td>
</tr>
<tr>
<td>PDB (100%)</td>
<td>35.46e</td>
<td>4.16d</td>
<td>8.49c</td>
</tr>
<tr>
<td>PDB (50%)</td>
<td>14.04g</td>
<td>3.72g</td>
<td>9.26e</td>
</tr>
<tr>
<td>PDB (25%)</td>
<td>3.34i</td>
<td>2.98e</td>
<td>2.76f</td>
</tr>
<tr>
<td>PDB (10%)</td>
<td>0.95i</td>
<td>2.81e</td>
<td>2.53f</td>
</tr>
</tbody>
</table>
Means with different letters in each column are significantly different from each other.

Table 3: Comparison of pathogens’ growth inhibitions by *T. asperellum* metabolite

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Means of growth inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. oxalicum</em></td>
<td>85.53a</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>72.67b</td>
</tr>
<tr>
<td><em>B. theobromae</em></td>
<td>48.26c</td>
</tr>
</tbody>
</table>

LSD<sub>0.05</sub> 10.25

Means with different letters are significantly different from one another at p<0.05

Figure 2: Total growth inhibition of *Botryodiplodia theobromae* by 100% *Trichoderma asperellum* metabolite
Mycoparasitic capability of *Trichoderma asperellum* and its metabolite

Figure 3: Total growth inhibition of *Penicillium oxalicum* by 100% *Trichoderma asperellum* metabolite
Figure 4: Growth inhibition of pathogens by *Trichoderma asperellum* metabolite

**Effect of *T. asperellum* metabolite on growths of *A. niger, B. theobromae* and *P. oxalicum***

There was total growth inhibitions on *A. niger, B. theobromae* and *P. oxalicum* by 100% concentration of the metabolite compared with their controls throughout the incubation period (Figures 1, 2 and 3). Growth inhibitions on the three fungi by the metabolite extracted by all the extraction solvents differed significantly (p< 0.05) from their controls (Tables 2). Inhibitions on the three fungi by *T. asperellum* metabolite differed significantly (p<0.05) from one metabolite concentration to the other. Inhibitions on the plant pathogenic fungi also increased significantly (p<0.05) with increase in *T. asperellum* metabolite concentration. For the three fungi, inhibitions by all concentrations of the metabolite extracted with N-Butanol were significantly (p <0.05) higher than others (Tables 2).

**Overall effect of *T. asperellum* metabolite on growths of the three fungi**

Generally, growth inhibitions of *P. oxalicum* and *A. niger* by the metabolite were significantly higher (p<0.05) than that of *B. theobromae*. Inhibition on *P. oxalicum* was also significantly higher (p<0.05) than that on *A. niger* (Table 3, Figure 4). In the dual culture of each of the fungi with *T. asperellum* (Table 1), growth inhibition on *B. theobromae* was also the least among the three fungi.

**DISCUSSION**

The action of *T. asperellum* against the growth of *A. niger, B. theobromae* and *P. oxalicum* showed the mycoparasitic potential of the *Trichoderma* species against a varying number of pathogens of different crops. This agrees with the work of Thiago *et al.* (2013) who reported that *T. asperellum* and *T. harzianum* possess promising mycoparasitic activities against plant pathogenic fungi and have a potential for biological control. The hyperparasitic capabilities of species of *Trichoderma* generally have been severally reported. For instance, the ability of spore suspensions of *T. harzianum* and *T. viride* to efficiently suppress the growth of *A. flavus* and *A. parasiticus* and also to reduce aflatoxin production in peanut kernels has been reported by Gachomo and Kotchoni(2008).

The effectiveness of the *T. asperellum* against the 3 fungi, both in dual culture and with the use of metabolite could be suggestive of the consistency of this fungus in its
Mycoparasitic attributes. Sharma and Sankaran (1988) and Johnsson et al. (1998) opined that a good antagonist must be able to demonstrate consistent efficacy against the target pathogen. The similarity of significant growth inhibition pattern of the three fungi by the metabolite with that obtained in dual culture might be underscoring the mycoparasitic consistency of *T. asperellum*. This may as well be suggestive of the promising antagonistic potential of the *Trichoderma* against the three fungi in vivo. The results also suggest that *T. asperellum* may possess more than one mechanism of antagonism. Agrios, (2005) stated that a microbial biological control agent may express different mechanisms against pathogens during their antagonistic activity. Rahman et al. (2009) reported that mycelial interaction is a basic method to assess antagonistic properties of microorganisms. The antagonistic potential of *T. asperellum* might be said to depend partly or wholly on its ability to compete for space and nutrients as well as its ability to produce antifungal compounds. *Trichoderma* species have been documented to control post-harvest diseases by production of volatile and non-volatile antibiotics (Dennis and Webster 1971, Tronsomo and Dennis, 1997). They are also known to be efficient producers of polysaccharide lyases, proteases, and lipases, all of which may be used for degradation of cell walls of target fungi. Chitinolytic and glucanolytic enzymes from *T. harzianum* for instance have been found to be inhibitory to other fungi and to interact synergistically to achieve an appreciable level of antifungal activity (Lorito et al., 1993). Yedidia et al. (2003) also submitted that *Trichoderma*’s growth inhibiting properties of other fungi are probably due to the combined action of cell-wall degrading enzymes together with the capacity of the *Trichoderma* to produce different secondary metabolites. Overall, the results obtained with the different concentrations of *T. asperellum* metabolite, suggests that a linear relationship may exist between metabolite concentration and growth inhibition of pathogen. Moreover, the significant growth inhibitions on the three fungi by the metabolite, even at lower concentrations compared to control underscores the possible antagonistic potential of the *Trichoderma* species against a wide range of pathogens by producing antifungal compounds. This agreed with the work of Chutrakul et al. (2008) who demonstrated the ability of *T. asperellum* to produce metabolites. Control of pathogenic fungi by the production metabolites has also been reported by different researchers (Ghisalberti, 2002, Howell, 2002, Spiegel et al., 2005, Intana et al., 2008, Khare et al., 2010).

Conclusively the result obtained in this study showed that *T. asperellum* is capable of inhibiting the growth of *P. oxalicum, B. theobromae* and *A. niger* hence could be used in the control of the tested fungi.

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