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Full Length Research Paper

Bioassay of five *trichoderma* strains against *Phytophthora megakarya* (*Cacao* pod-rot) in Nigeria

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Bioassay of five *Trichoderma* strains isolated from Cocoa ecological zones of Nigeria was carried out at Cocoa Research Institute of Nigeria. In the *in vitro* assessment the pathogen and antagonists were paired on potato dextrose agar (PDA) contained in 90 mm Petri dishes, while the effect of antagonists on the mycelial growth of the pathogen was determined for 120 h. The percentage disease incidence of the pathogen in the presence of the antagonists was determined using cocoa pod husk pieces (CPHP) and the early infection index of the pathogen was calculated over 8 days. All the *Trichoderma* strains screened inhibited the growth of the pathogen exhibiting between very strong (6) and strong antagonism (5) on an assessment scale of 0 to 6. The inhibition increased daily till the pathogen disappeared. The presence of *Trichoderma* strains reduced disease from 95.0% in the control to 25.0% the treatment that had highest incidence. The Early Infection Index (EII) was low in all the treatment in relation to the control. The highest EII (16.03%) which was recorded in NIG-T293 treated samples was very low compared with the control (100%). The screened fungi are therefore potential bio-agents capable of controlling the disease on the field.

Key words: Bioassay, *Trichoderma* strains, disease incidence, biological control.

INTRODUCTION

The importance of Cocoa to Nigerian economy is notable in the area of foreign exchange earning as the crop has contributed considerable amounts to the capital savings of the country. Between 1959 and 1969, average incomes of N78 to N106 m per annum (19 – 40% of the total annual value for all exports from Nigeria) were realized from Cocoa sale in form of foreign exchange earnings (FOS, 1972).

However, the production of Cocoa has been on decline due to factors such as rural to urban drift, government polices and most importantly pest and disease outbreaks (Pan, 2001). Since the beginning of *Cacao* cultivation, the fungal disease has remained the major limiting factor of production both quantitatively and qualitatively (Evans et al*.,* 1998). Recent reports have however shown that in Nigeria losses up to 100% have been reported with some virulent strains of *Phytophthora megakarya* (Agbeniyi and Adedeji, 2003a). There has been consistent downward trend in Nigerian cocoa production and position in the world market (Sanusi and Oluyole, 2005). Nigeria has dropped from the initial second position to forth largest producer after Cote d'Ivoire, Ghana and Indonesia (International Cocoa Organisation, 2003).

Chemical control has been the most effective for decades (Agbeniyi and Adedeji, 2003b); however the efficacy of fungicides has been reduced by the development of resistance among pathogens exposed continuously to the chemicals and deterioration of environmental quality (Poincelot, 1986). Chemical control method is expensive for the small holders as it costs a lot of money to hire labour and purchase machine and chemicals (Gorenz and Okaisabor, 1971) which makes it unsustainable economically (PAN, 2001), hence the search for alternative to chemicals.

Biological control of plant disease, on the other hand lacks most of the above limitations but it is safe and sustainable (Cook and Baker, 1983; Chet, 1987; Janisiewiez and Korsten, 2002; Spadaro and Gullino, 2005). *Trichoderma* species have potentials as biological control agents against many plant pathogens (Kubicek et al., 1996; Tronsmo and Hjeljord, 1998; Harman, 2000). Some strains of *Trichoderma* had been identified as potential

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biological control agents of plant pathogenic fungi on many crops including pepper (Odebode and Sobowale, 2001), onion (Abd-el Moity et al., 1982), maize (Sobowale et al., 2005) and cotton (Ozbay and Newman, 2004). The exploration of biological control strategy against *Phytophthora* pod-rot of cocoa therefore be-comes a viable option in view of its reported effectiveness in the control of various plant diseases. This study is therefore aimed at screening five *Trichoderma* strains for their antagonistic activities against *P. megakarya* for further development to biological control package**.**

EXPERIMENTAL

Isolation and identification of bio-agents

The antagonists used were isolated from cocoa pods by scrapping their surface up to 20 mm deep. One gram each was macerated in 10 ml of sterile distilled water with sterile mortar and pestle. One ml each of the macerated samples was pipetted per plate and spread evenly onto PDA inside 90 mm diameter Petri dishes. Three replicate plates were used per sample and incubated at 25 ± 2°C for one week. Using sterile inoculating needle, three plates were inoculated with similar colony types in order to purify them on PDA plates. Pure culture of each fungus was stored in slant under sterile distilled water in McCartney bottles for future use. Similar colony types were sub-cultured onto pure PDA plates. Using sterile inoculating needle, three plates were inoculated per colony. Pure culture of each fungus was tentatively identified using a guide by Samuels (2001). Samples from the pure cultures were then sent to USDA Systematic Botany and Mycology Laboratory, United State of America (USA) for confirmation. Parts of the samples were equally stored in slant under sterile distilled water inside McCartney bottles at 10°C for future use.

Isolation of *P. megakarya*

The pathogen, *P. megakarya* was isolated from naturally infected cocoa pods. The pods were surface sterile with 75% alcohol. Two-meter cube pieces were removed using sterile scalpel and aseptically placed on PDA plates. The plates were incubated under 22 ± 2°C for 7 days. Sub-culturing was done to obtain pure cultures.

Screening for antagonistic activities *in vitro*

The experiment was laid out in a complete randomized design (CRD) with six treatments and three replicates. The treatments were the five bio-agents versus the pathogen and the 6th is the control. Three sets of six Petri dishes receiving the same treatment served as replicate. Each bio-agent was paired against *P. megakarya* on PDA contained in 90 mm diameter Petri dishes following a method described by Odebode and Sobowale (2001): (i) Five millimetre plug of each of the bio-agents was placed at 20 mm away from one edge of 90 mm Petri dish and after 24 h *P. megakarya* was placed at 20 mm away from the other edge of the same Petri dish, (ii) *P. megakarya* was placed at 20 mm away from one edge of the Petri dish and after 24 h the bio-agent was placed at 20 mm away from the other edge of the same Petri dish (iii) the bio-agent and *P. megakarya* were inoculated at the opposite edge of the Petri dish simultaneously. The set up was incubated at 22 ± 2°C for 10 days, while observation on colony growth of both bio-agents and pathogen were done every 24 h taking two radial growth at right angle to one another and find the average measurements.

Evaluation of bio-agent activity was done using the modified scale of Turhan and Grossman (1994).

Determination of percentage infection and early infection index of *Phytophthora megakarya* on pre-treated cocoa pod husk pieces

This experiment was designed to screening the bio-agents for their ability to prevent the pathogen from infecting cocoa pods using cocoa pod husk pieces (CPHP). The method has been reported to take care of the short coming of *in vitro* screening (Tondje et al., 2006). The experiment was laid out in a complete randomized design (CRD) with five treatments and three replicates. Cocoa pod husk pieces (CPHP) were prepared by cutting through the husk with 6 mm diameter disc. Six of the discs were placed in a 90 mm diameter Petri dish. Five dishes were used for the experiment. The CPHP were pre-treated by spreading a 50 µl drop of 1.0 x 10^6 spores' ml^{-1} of bio-agents on to the upper surface of the CPHP to facilitate the pre-colonization process. For the control, 50 µl of sterile distilled water was spread on the upper surface of the CPHP. The plates were covered and incubated at room temperature (29 ± 2°C) for 4 days. During incubation period, moisture was provided by regularly adding sterile distilled water to facilitate germination and growth on CPHP surfaces of the bio-agents. The CPHP were transferred to new Petri dishes and allowed to air dried under aseptic condition for 3 h. A 15 µl drop of zoospore suspension of the pathogen at a concentration of 3.0 x 10^5 zoospores ml^{-1} were inoculated to the centre of completely dried surface of each CPHP. First observations were made two days after inoculation and then every other day for 8 days. Early infection index (EII) on CPHP was calculated using a formula adapted from Tondje et al., (2006) thus:

$$EII = 6X_2 + 4X_4 + 2X_6 + X_8,$$

Where X = % Infection recorded on CPHP on days 2, 4, 6 and 8,

$$X = [(C - T)/C] \times 100$$

Where C = Total CPHP infected in the control, and T = Total CPHP infected in the treated.

RESULTS AND DISCUSSION

Antagonistic activities of bio-agents against *P. megakarya in vitro*

In the *in vitro* assessment of antagonism, it was observed that all the *Trichoderma* strains screened inhibited the growth of the pathogen. The ability of bio-agents to inhibit the pathogen growth increased as day of inoculation increased. At 120 h after inoculation (HAI), all the bio-agents had between strong antagonism (5) and very strong antagonism (6) on an assessment scale of 0 to 6 on the pathogen during *in vitro* screening (Table 1). At the the point of contact, the bio-agents were observed to be overgrowing the pathogen. The pathogen on the other hand was continuously reducing in size until it finally disappeared. This could be due to the fact that the bio-agents were feeding or parasitizing on the pathogen, an act referred to by Boosalis and Mankau (1965) as mycoparasitism. NIG-T287 demonstrated more antagonism than all other bio-agents by exhibiting very strong antagonism (6) in the three inoculating methods (Tables

Table 1. Antagonistic activity of five *Trichoderma* strains when inoculated 48 h after *P. megakarya* inoculation.

Trichoderma strain	Antagonism				
	24HAI*	48HAI	72HAI	96HAI	120HAI
NIG-T287	2	2	3	5	6
NIG-T288	1	2	2	4	5
NIG-T289	1	1	2	4	5
NIG-T290	1	2	3	4	5
NIG-T293	1	2	3	5	5

NIG-T287 = *T. asperellum*, NIG-T288 = *T. asperellum*, NIG-T289 = *T. harzianum*, NIG-T290 = *T. asperellum,* NIG-T293 = *T. asperellum*, Pm = *Phytophthora megakarya*.
*Hours after inoculation.

Table 2. Antagonistic activity of five *Trichoderma* strains when inoculated 48 h before *P. megakarya* inoculation.

Trichoderma strain	Antagonism				
	24HAI*	48HAI	72HAI	96HAI	120HAI
NIG-T287	2	4	4	5	6
NIG-T288	2	3	4	5	6
NIG-T289	1	2	3	5	6
NIG-T290	2	3	3	5	6
NIG-T293	2	3	4	5	6

NIG-T287 = *T. asperellum*, NIG-T288 = *T. asperellum*, NIG-T289 = *T. harzianum*, NIG-T290 = *T. asperellum,* NIG-T293 = *T. asperellum*, Pm = *Phytophthora megakarya*.
*Hours after inoculation.

1, 2 and 3). This observation however disagreed with opinion of Campbell (1989) that bio-control agents are more active against pathogens when they are first inoculated on the host since there are no bio-control agents that have enough competitive ability to displace an already established pathogen. Inoculation of bio-agents 24 h before the pathogen (Table 1) favoured them to have very strong antagonism (6) as against the strong antagonism (5) they had when inoculated 24 HAI and simultaneous inoculation (Tables 2 and 3). This finding however agreed with works of Roberts (1990) and Odebode and Sobowale (2001) that the time lapse between inoculation of antagonist and the pathogen contributed to the success normally recorded with the antagonist against the pathogen.

Percentage infection and early infection index of *P. megakarya* on pre-treated cocoa pod husk pieces (CPHP)

Since the validity of simple agar plate method of screening bio-agents antagonistic activities has been questioned by various workers (Tronsmo, 1992), the antagonistic activity was screened using cocoa pod husk pieces (CPHP). Table 4 shows the percentage infection by days for eight days and the Early Infection Index (EII) (%) of *Phytophthora* pod-rot on cocoa pod husk pieces (CPHP) pre-treated with conidial suspensions of *Trichoderma* strains. In the presence of *Trichoderma* strains the highest percentage infection for the period of eight days was 25.0%. This was recorded on NIG-T290 treated samples, while the control recorded 95.0% infection. Ten percent infection each was recorded on NIG-T287 and NIG-T288 treated CPHP and 15% each was recorded on NIG-T289 and NIG-T293 treated CPHP, respectively.

Though none of the bio-agents completely prevented pod-rot symptom on the CPHP, however the Early Infection Index (EII) was low in relation to the control. This is an indication that these bio-agents would reduce the disease on the field. The highest EII (16.03%) which was recorded in NIG-T293 treated samples was low compared with the control (100%). This is however different from the *in vitro* result where in NIG-T293 had very strong antagonism even when it was simultaneously inoculated with the pathogen. The least Early Infection Index (3.05%) was recorded on NIG-T288 treated CPHP. The advantage of bioassay with CPHP has been emphasized by Tondje et al. (2006) that since the pod is the

Table 3. Antagonistic activity of five *Trichoderma* strains against *P. megakarya when* they were simultaneously inoculated.

Trichoderma strain	Antagonism				
	24HAI*	48HAI	72HAI	96HAI	120HAI
NIG-T287	2	3	4	5	6
NIG-T288	1	2	3	4	5
NIG-T289	1	1	2	3	5
NIG-T290	1	2	3	4	5
NIG-T293	2	2	4	5	6

NIG-T287 = *T. asperellum*, NIG-T288 = *T. asperellum*, NIG-T289 = *T. harzianum*, NIG-T290 = *T. asperellum,* NIG-T293 = *T. asperellum*, Pm = *Phytophthora megakarya*.
*Hours after inoculation.

Table 4. Percentage infection and early infection index (EII) of pod-rot on cocoa pod husk pieces (CPHP) pre-treated with *Trichoderma* strains.

Treatment	CPHP Infected by day (%)				*Early Infection Index (%)
	Day 2	Day 4	Day 6	Day 8	
NIG-T287 + Pm	0.00	0.00	10.00	10.00	***4.58
NIG-T288 + Pm	0.00	0.00	5.00	10.00	3.05
NIG-T289 + Pm	0.00	5.00	15.00	15.00	9.92
NIG-T290 + Pm	0.00	10.00	10.00	25.00	12.98
NIG-T293 + Pm	5.00	5.00	10.00	15.00	16.03
Pm only (Control)	25.00	60.00	85.00	95.00	100.00

NIG-T287 = *T. asperellum*, NIG-T288 = *T. asperellum*, NIG-T289 = *T. harzianum*, NIG-T290 = *T. asperellum,* NIG-T293 = *T. asperellum*, Pm = *Phytophthora megakarya*.
***Values are expressed (%) to control.

the primary site of pod-rot infection the result obtain would be very close to the actual expression on the field. Several endophytes have been reported to support growth and improve the health of plants (Kirchhof et al., 2001). These could as well be important sources of bio-logical control in cocoa (Arnold et al., 2003) however, the search for bio-agents against pod-rot pathogen is at the pioneer stage in Nigeria, the next stage is therefore to apply these bio-agents on the field.

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