American-Eurasian Journal of Agronomy 5 (2): 30-34, 2012
ISSN 1995-896X

DOI: 10.5829/idosi.aeja.2012.5.2.2046

Studies on *Penicillium digitatum, Botryodiploidia theobromae, Alternaria tenuis* and *Trichoderma harzianum* for Bicontrol of *Phytophthora palmivora* Cocoa Black Pod Disease Pathogen

[1]M.O. Adebola and [2]J.E. Amadi

[1]Department of Biology Sciences, Ibrahim Badamasi Babangida University, Lapai, Nigeria
[2]Department of Botany, Nnamdi Azikiwe University, Awka, PMB 5025, Awka, Nigeria

Abstract: *In vitro* studies on biological control of *Phytophthora palmivora* cocoa black pod disease pathogen by *Penicillium digitatum, Botryodiploidia theobromae, Alternaria tenuis* and *Trichoderma harzianum* were carried out using dual culture technique. The percentage inhibition of radial growth of the pathogen was assessed and the width of the zone of inhibition was measured. All data were analysed using analysis of variance and Duncan Multiple range test. The results revealed a significant difference ($P<0.05$) in radial growth of pathogen when in dual culture with tested fungi and when it was alone. *P. digitatum* and *Trichoderma harzianum* grew faster, effectively checked the growth of *P. palmivora* and produced zones of inhibition. *B. theobromae* and *Alternaria tenuis* did not show enough potential. The inhibitory effect of the culture filtrates of potential antagonists on pathogen was significant ($P < 0.05$) and mostly began on day 5 and increased with age of incubation. *Trichoderma harzianum* and *P. digitatum* were observed to be effective at checking the growth of *P. palmivora* in both solid and liquid media and therefore showed the potentials for the biological control of the pathogen. while *A. tenuis* and *B. theobromae* did not show enough potential under the conditions of this study.

Key words: Cocao % Biocontrol % Pathogen % Antagonists % Inhibition

INTRODUCTION

Cocoa (*Theobroma cacao* L.) family *Sterculiaceae* is subject to numerous constraints due to plant and animal parasites or viruses. Among the diseases cause by these parasites are: black pod disease (*Phytophthora palmivora;* cocoa swollen shoot (viral); *Monilia* pod rot (*Moniliophthora roreri*); witches broom (*Crinipellis* perniciosa); *Botryodiploidia* pod rot (*Botryodiploidia theobromae*); collar crack (*Armellaria mellea*); cushion gall (*Calonetria rigidiuscula*) [1, 2]. Of all the plant pathogens, *Phytophthora*; the 'plant destroyer' is one of the most destructive fungal genera in temperate and tropical regions, causing annual damages of billions of dollars. Throughout the world most damage is caused by *P. palmivora* and the impact of the disease varies from country to country [3]. Direct crop losses of up to 90% occur in wetter areas such as Nigeria [4]. Curative measures for the disease have not been successful. All efforts are therefore directed towards preventing the incidence of the disease. Frequent harvesting and frequent removal of infected pods will generally reduce black pod but are not practiced as much as they should. [5, 6]. The use of resistant varieties of cocoa is apt to breaking down under adverse weather condition or from the appearance of new strain of the pathogen. Breeding resistant cocoa variety is more laborious and most varieties now grown are susceptible to *Phytophthora.* It will therefore be difficult to obtain completely resistant cultivars. Moreover, according to Tondje *et al*. [7] no cocoa variety is completely resistant to black pod disease caused by *Phytophthora.*

Following from these, alternative or complimentary methods are needed for management of this disease. Biological control comes in handy here [8, 9]. For instance, the necrotroph *Trichoderma* has the capacity to parasites a great number of fungi, including pathogens *Rhizoctonia*, *Scerotinia*, *Fusarium* and *Verticillium* and the Straminopiles *Pythium* and *Phytophthora*. *Trichoderma* grows towards hyphae of other fungi where

Corresponding Author: M.O. Adebola, Department of Biology Sciences, Ibrahim Badamasi Babangida University, Lapai, Nigeria

it binds following branching. *Trichoderma* releases hydrolytic enzymes that digest the walls of the host prior to penetration. *Trichoderma* is also thought to release a range of toxins that reduce any response from the host to invasion [10, 11]. The goal of this study is to select the good biocontrol fungi that can suppress *Phytophthora palmivora*, the cocoa black pod disease causative organism in the field. A preliminary test to identify competent antagonists that inhibit disease cause by this pathogen would greatly accelerate the selection process and the success will be a major breakthrough.

MATERIALS AND METHODS

Isolation of Pathogen and Potential Antagonistic Fungi: The native potential antagonistic fungi were isolated from cocoa rhizosphere and rhizoplane in farmers' fields at Aba Ijesha in Atakunmosa L.G.A. Osun State Nigeria. The leaves, stems and roots taken from healthy and infected cocoa trees were surface sterilized with sequential washes in tap water, 95% ethanol, commercial sodium hypo chlorite, 75% ethanol and sterilized distilled water [8, 9]. To allow for the growth of all organisms, the materials were placed on water agar and incubated at room temperature (28 ± 2°C) for 7 days [7]. Isolation was also made from soil using serial dilution method [12]. The pathogen (*Phytophthora palmivora*) was isolated from freshly infected cocoa pods obtained from Cocoa Research Institute of Nigeria (CRIN), Ibadan. Infected pods were surface sterilized with 70% ethanol, the epidermis was stripped off with a sterile scalpel and the pod tissue was plated directly on Petri dish containing water agar and incubated at room temperature (28 ± 2°C) for 72hrs after which subcultures were made on PDA [12, 13]. Stock cultures of isolates were maintained at 4°C for subsequent studies.

Screening the Isolates for Antagonism: Inhibition of *Phytophthora palmivora* growth on PDA by the two test fungal isolates was evaluated using dual culture technique. Five millimeter diameter mycelial plugs of each of the potential antagonists were placed at the periphery of three replicates culture plates and incubated for 2 days at 28±2°C [2, 9, 14]. A mycelial plug (5mm diameter) of *P. palmivora* then was placed 5cm from inoculums point of the potential antagonist and the plates were incubated for an additional 9 days at 28 ± 2°C. The growth, in millimeter, of the *P. palmivora* and the potential antagonists were recorded. The control was made using sterile agar in place of the potential antagonist. The growth of pathogen and the potential antagonists was recorded. Percentage inhibition of radial growth was assessed (100 x $(R_1 - R_2)/R_1$) where R1and R2 were the radial growths of the pathogen in sole culture (control)and in dual culture with potential antagonist respectively and the width of the zone of inhibition (ZI) measured at the smallest distance between both colonies in dual culture plate [8, 15, 16, 17].

Antagonism of Potential Antagonists Filtrates on Pathogen: One hundred ml of Potato Dextrose Broth (PDB) in Erlenmeyer flask (250ml) were separately inoculated with a disc of 5mm -diameter punched out from the edge of a 7-day- old colony of potential antagonist grown on PDA. Each flask was inoculated with three discs and set up incubated at 28 ± 2°C for7, 14, 21and 28 days. Culture filtrates were harvested by filtering through Whatman No. 1 filter papers. Nine millimeters (9ml) of each test filtrate were used to amend 50ml of sterile PDB. The amended broth was then inoculated with a 5-mm-diameter mycelia plug of the pathogen. The control flasks were amended with 9ml of sterile distilled water. Each test was replicated three times. Inoculated flasks were incubated at 28 ± 2°C for 7days after which the mycelia in each flask were harvested, dried to constant weight in an oven and weight recorded. The percentage inhibition was calculated [9, 17, 18]. All data were statistically analyzed using ANOVA and Duncan's Multiple Range Test (DMRT) [8, 13].

RESULTS

Four potential antagonistic fungi *Alternaria tenuis*, *Trichoderma harzianum*, *Botryodiploidia theobromae* and *Penicillium digitatum* were isolated and identified from cocoa phylloplane and rhizosphere. The pathogen (*Phytophthora palmivora)*) was isolated from freshly infected pods collected from CRIN, Ibadan Nigeria.

Antagonism in Solid Medium: The result of the experiment (Table 1) showed that the two tested fungi exhibited antagonistic activities on the pathogen. There was a significant difference ($P < 0.05$) in radial growth of pathogen in dual culture with tested fungi and the control. The potential antagonists except *Alternaria tenuis* were observed to grow faster and higher with days of incubation in dual culture with the pathogen. There was significant difference ($P < 0.05$) in percentage inhibition of radial growth of pathogen in dual culture with the potential antagonists. The percentage inhibition was 56% with *B. theobromae* 74% with *Penicillium digitatum*,

Table 1: Radial growth (mm), percentage inhibition and zone of inhibition of pathogen in dual culture with potential antagonists

Dual culture of Pathogen with	* Mean radial Growth (mm) of Pathogen	*Mean % inhibition Of pathogen	*Mean zone of Inhibition
Alternaria tenuis	27c	35a	13b
T. harzianum	10a	76d	16c
B. theobromae	17b	56b	11a
P. digitatum	10a	74c	15c
Control	39d	-	-

* Mean of three replicates at 9 days of incubation

Means in a column followed by different letters differ significantly at P < 0.05 (DMRT)

Table 2: Effects of ages of potential antagonist culture filtrates on % inhibition of pathogen growth.

Age (day)	*Mean % inhibition of pathogen
5	35.5a
15	38.7ab
25	42.0b
35	49.5c
45	61.9d

*Mean of all the potential antagonist culture filtrates

Means in a column followed by different letters differ significantly at P< 0.05 (DMRT)

Table 3: Percentage inhibition of pathogen growth in potential antagonist culture filtrates

Culture filtrate of antagonist	*Mean % inhibition of pathogen
Alternaria tenuis	30a
T. harzianum	54d
B. theobromae	39b
P. digitatum	44c

*Mean of all the ages of culture filtrates

Means in a column followed by different Letters differ significantly at P< 0.05 (DMRT)

35% with *Alternaria tenuis* while it was 76% with *T. harzianum* (Table1). There was significant difference (P<0.05) in Zone of inhibition produced by the potential antagonists. However, it was not significantly different between *T. harzianum and P. digitatum. T. harzianum* produced the largest zone of inhibition (16mm) while *B. theobromae* produced narrowest zone of inhibition (11mm) (Table 1). The zone of inhibition was also found to decrease with increase in days of incubation. There was significant different (P < 0.05) in zone of inhibition among the days. It was observed that after day 9 there was little or no distinct zone (gap) left between the pathogen and potential antagonist.

Effects of Culture Filtrate on Antagonism: The results showed generally that the inhibitory effect of the culture filtrates of potential antagonists on pathogen was significant (P < 0.05) and mostly began on day 5 and increased with age of incubation (Table 2). The inhibitory effect produced in culture filtrate of *T. harzianum* increased rapidly with the age of the culture reaching the average of 54% inhibition on day 45 of incubation. In *Alternaria tenuis* it was 30% (Table 3).

DISCUSSIONS

The goal of this study is to select the good biocontrol fungi that can suppress *Phytophthora palmivora*, the cocoa black pod disease causative organism in the field. A preliminary test to identify competent antagonists that inhibit disease cause by this pathogen would greatly accelerate the selection process. Out of four potential antagonistic fungi isolated, only two were able to challenge the pathogen effectively in vitro. The screening of the potential biocontrol fungi isolated from the cocoa farms was in line with the report of Bong *et al*. [3]; Kamil and Yahya, [19] who pointed out that a lot of beneficial fungi and bacteria that occur naturally and associated with cocoa in Malaysia showed potential as antagonists of major cocoa pathogens. Samuel and Hebbar, [20] advised that effort and expense in finding novel biological control species and strains can be saved by directing the search either by exploration for new strains in native area of the host or its pathogen (classical biological control technique) or by studying the species that are known to be phylogenetically related to species that is effective in biological control (molecular phylogenetics). The dual inoculation and the introduction of potential antagonists two days before the pathogen was in agreement with the work of Campbell, [21] who believed that there are no biocontrol agents that have enough competitive ability to displace an already established pathogen. The time lapse between inoculation of the antagonist and the pathogen contributed to the success recorded with the antagonist against the pathogen. This is similar to the observation of Robert, [22] and Janisienwicz, [23] on the importance of time lapse between the arrival of the antagonist and later pathogen on the phylloplane. According to them this allows adequate increase in cell concentration and subsequent colonization by antagonist before the arrival of the pathogen.

The result of this study showed that *T. harzianum and Penicillium digitatum* grew faster than the pathogen in dual culture on solid medium. This partly confirmed the report of Adejumo *et al*. [24] that *Trichoderma sp* are fast growing fungi and as such exhausted available nutrient for growth without coiling or distortion of the hyphae of the pathogen. According to Dandurand and Knudsen, [25] the effectiveness of biocontrol agents might depend partially on their ability to proliferate during a short period of favorable environmental conditions before they encounter plant pathogen. More rapid growth and sporulation of fungi from biocontrol formulations may superficially enhance efficacy in the field. Going by Turhan and Grossman, [26] modified scale, *Trichoderma harzianum* and *Penicillium digitatum* showed strong antagonism while *B. theobromae* showed moderate antagonism *and Alternaria tenuis is a* weak antagonist. The tested fungi produced zone of inhibition at the point of contact with the pathogen. This confirmed the report of Royse and Ries, [15] that in vitro fungal interactions resulted in one of the following; Production of a zone of inhibition (ZI), contact inhibition or no inhibition. The zones of inhibition produced might be due to the production of antifungal metabolites produced by the tested fungi as reported by Shanker *et al.* [27].

The results from the culture filtrate of the tested potential antagonists showed that *T. harzianum* produced higher antifungal activity with 54% inhibition of pathogen growth. However, none of the potential antagonists culture filtrates completely inhibit the growth of *P. palmivora* supporting the report of Corinne *et al*. [28] that none of the filtrates of the two tested antagonists was able to completely inhibit the growth of the *P. palmivora*. The effect of the culture filtrate on pathogen growth that increased with the days of incubation was similarly reported by Odigie and Ikotun, [12] on antifungal activity of *G. roseum* filtrate on *P. palmivora* which increased between day 3 and 30 of incubation. Out of the two potential antagonistic fungi isolated, only *T. harzianum and Penicillium digitatum* were able to challenge the pathogen effectively *in vitro*. This result confirmed the report of Bong *et al*. [3]; Kamil and Yahya, [19] who pointed out that a lot of beneficial fungi and bacteria that occur naturally and associated with cocoa showed potential as antagonists of major cocoa pathogens.

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