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IN VITRO SCREENING OF MEDIA AND OTHER FACTORS ON BIOLOGICAL CONTROL OF COCOA BLACK POD FUNGUS (*PHYTOPHTHORA PALMIVORA*)

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SUMMARY

Cocoa black pod pathogen(*Phytophthora palmivora)* cause an annual crop losses that may range from 30 - 90% in cocoa farms. Therefore, *in vitro* screening was undertaken to assess the effects of media and other factors on potential of four fungi *(Paecilomyces sp, Trichoderma harzianum, Rhizopus stolonifer and Penicillium digitatum)*as biological control agents against this pathogen. The test fungi were isolated from cocoa farm and the screening was done on M9 medium using dual culture techniques. The results showed that the six media tested enhanced antagonistic activities and could be good for the study of biological control. The percentage inhibition of pathogen growth observed in each medium was well above 60%. Temperature range for the antagonistic activities of the fungal isolates was between 15 – 35°C outside which the antagonistic activities dropped. The effect of pH range used was observed to be significantly ($P<0.05$) decreased with increase in pH. Strong antagonism of the pathogen was observed between pH 5 – 6.5. The result from nutritional factors on antagonistic activities of the four fungal isolates revealed that the antagonistic effects were not significantly different ($P<0.05$) among the supplements used but was highest in pectin- and glucose- supplemented media suggesting that nitrogen and carbon sources were essential for successful production of inoculum biomass as well as for sustaining biological activities. The efficacy of these tested fungi under field conditions is further being investigated.

Key words: Nutritional factors; Environmental factor ; Antagonism; *Phytophthora palmivora*

Black pod disease pathogen (*Phytophthora palmivora*) of cocoa (*Theobroma cacao* L. family Sterculiaceae) caused an estimated loss in production in Asia, Africa and Brazil of 450,000 tonnes annually, worth an estimated value of $423million. Annual crop losses range from 30 - 90% (3). Curative measures for the disease have not been totally successful(2 and 3). Earlier studies had shown that the use of chemical is very expensive, uneconomical, caused some burning of the young, tender leaves, stunted growth, pod injury, loss of biodiversity, spoilage of land and water (8,14,5). Recent

researches showed that biological control approach using antagonistic microorganisms could be one of the alternatives or complementary methods for management of this disease. This offers the possibility of a less expensive and more environmenta sensitive control strategy.

Success in the use of biological control had been reported. It was reported that the growth and antagonistic activity of fungi may be influenced by nutritional and environmental conditions(15). Laboratory assays for antagonism using the dual culture method of (19) indicated that the antagonism differed when tested in different media and affected by nutritional factors. It had earlier been reported that the i*n-vitro* potentials of *Aspergillus fumigatus, A. repens, A. niger, Paecilomyces sp and Rhizopus stolonifer* at checking the growth of *Phytophthora palmivora* (1.2). It is on this note, that this research was directed at investigating the *in-vitro* effect of environmental and nutritional factors on the potentials of *Paecilomyces sp, Trichoderma harzianum, Rhizopus stolonifer and Penicillium digitatum* at checking the growth of *Phytophthora palmivora.*

MATERIALS AND METHODS

Isolation of potential antagonists and pathogen

Paecilomyces sp, Trichoderma harzianum, Rhizopus stolonifer and Penicillium digitatum were isolated from cocoa rhizosphere and rhizoplane in farmers' fields at Aba- Ijesha in Atakunmosa L.G.A. Osun State Nigeria. The organisms were identified at the Mycology unit, Microbiology Department, University of Ilorin Nigeria. The pathogen (*Phytophthora palmivora)* was obtained from Cocoa Research Institute of Nigeria (CRIN), Ibadan.

Influence of media

Effect of media on parasitism was assessed using the following media: carrot meal agar, malt extract agar, cassava dextrose agar, corn meal agar and Potato Dextrose Agar. Three replicates of each were made. Mycelia-plugs of 5mm diameter each were cut from the edge of actively growing antagonist culture and placed at the periphery of the culture plates and incubated for 2 days at 28 ± 2°C. A mycelia-plug (5mm) of *P. palmivora* was placed 5cm from inoculum point of the antagonist while the control was not challenged. The plates were incubated for an additional 9 days at 28 ± 2°C after which the radial growth in mm of the pathogen and the antagonist were measured and recorded. The percentage inhibition of radial growth of pathogen was determined ($100 \times (R_1 - R_2)/R_1$) where R1and R2 were the radial growths of the pathogen in sole culture (21; 2) and results were analysed using Analysis of Variance(ANOVA)and Duncan Multiple Range Test(DMRT)

Effect of nutrition

This was assessed on M9 medium with the following composition; NaH_2PO_4 (6g); KH_2PO_4 (3g); NaCl (0.5g); NH_4Cl (1g); and distilled water (1 litre) (15). The medium with agar was autoclaved and then supplemented with sterile 1M $MgSO_4.7H_2O$ (2 ml); 20% glucose (10ml); 1M $CaCl_2$ (0.1ml) and 1% vitamin B (thiamine-HCl) 0.5ml. The carbon

and nitrogen sources were added to M9 medium separately after autoclaving. The eight carbon sources lactose, pectin, glucose, sucrose, starch, fructose, maltose and galactose were added to the M9 medium at a final concentration of 0.2% (w/v) each being substituted for glucose. Similarly, the eight nitrogen sources methionine, lysine, urea, $Ca(NO_3)_2$, $NH_4H_2PO_4$, NH_4Cl, KNO_3, and $NaNO_3$ were incorporated into the M9 medium at a final concentration of 0.2% (w/v) each being substituted for NH_4Cl. Antagonism was assayed on the carbon and nitrogen sources using the dual method of (19). Cultures of the antagon-ists and pathogen were inoculated on M9 medium plates at fixed positions, 5cm apart while the control was not challenged. The plates were incubated at 28 ± 2°C and the growth of pathogen in each of the plates was measured at 24 hours intervals for 7 days (15). The percentage inhibition of the pathogen radial growth was calculated (100 x $(R_1 - R_2)/R_1$) where R1and R2 were the radial growths of the pathogen in sole culture (21;1). There were three replicates for each treatment and the results were analysed using ANOVA and DMRT.

Effect of pH and temperature

The pH range (pH 5.0 to 8.0)tested was ass essed using the dual culture technique on ste-rilized M9 medium adjusted with sodium hyd-roxide (NaOH) or hydrochloric acid(HCl).

The culture plates were doubly-ino culated while control was not challenged and incubated for 9 days at 28 ± 2°C. The M9 medium as described above was also used to assess the effect of temperature. Doubly-inoculated culture plates were incubated at 15, 20, 25, 30 and 35 °C. Three replicates were made for each treatment and the growth of the pathogen and the antagonist were recorded. The percentage inhibition of growth was determined and the results were analysed using ANOVA and DMRT (15,2)

RESULTS

Effect of media on parasitism

The result revealed that the tested media influenced the activities of the potential antagonists significantly ($P < 0.05$) (Table 1). Malt extract agar enhanced the strongest (70.0%) growth inhibition,followed by PDA(68.1%), corn meal agar (67.4%), cassava dextrose agar (67.1%) and carrot meal agar (66.1%).

Effects of nutritional factors

Variation in antagonistic activities was observed among the potential antagonists in the eight carbon sources tested in M9 medium. The highest antagonism was observed in pectin-supplemented medium except for *Penicillium digitatum* that utilized glucose followed closely by media supplemented with glucose, fructose and sucrose in that order. However, the antagonistic effect was not significantly different among these supplements ($p < 0.05$). *T. harzianum and R. stolonifer* were able to utilize galactose and lactose very well but the least utilized by other test organisms (Fig

2). The eight nitrogen sources tested also affected the antagonistic activities of the test antagonists. The strongest antagonism (79%)was shown in $NH_4H_2PO_4$ supplemented medium followed by $Ca(NO_3)_2$ (76%), NH_4Cl (72%)and urea (71%). There was no significant difference ($P < 0.05$) observed in the utilization of methionine, $NaNO_3$ and KNO_3 supplemented media by all the test antagonists. It was observed that *T. harzianum* gave the strongest antagonism in all the nitrogen sources (Fig 2).

Effect of environmental factors

The effect of pH range on antagonism was observed to be significant ($P<0.05$) (Table 3). The antagonistic activities decreased with decrease in pH. *T. harzianum* produced the strongest inhibition among the test organisms as the acidity increases.

Temperature significantly influenced ($P<0.05$) the antagonistic activities of the test antagonists (Table 4). Inhibition of the pathogen was observed in all the ranges of the temperature (15 – 35°C). Inhibition was not significant between 20°C to 30°C but maximal at 30°C. At 35°C the inhibition was observed to be very low. Antagonist *T. harzianum* produced the strongest inhibition at all the ranges of temperature.

glucose and acetate only at pH 6.8 whereas at pH 3.4 the cells were permeable to all the

Table 1:Mean effects of media on antagonistic activities of test fungi

Medium	* % Inhibition pathogen grown
Carrot meal agar	66.1b
Cassava dextrose agar	67.1b
Corn meal agar	67.4b
Potato dextrose agar	68.1b
Malt extract agar	70.0b

*Mean of three replicates after 9 days of incubation
Means in a column followed by different letters differ significantly at P<0.05 (DMRT)

Table 2:Mean effects of pH on antagonistic activities of potential antagonists on a pathogen in dual culture on M9 medium

pH	* % Inhibition
8.0	41.5a
7.5	43.9ab
7.0	48.9b
6.5	54.9c
6.0	62.4d
5.5	70.1e
5.0	71.6e

*Mean of three replicates after 7 days of incubation
Means in a column followed by different letters differ significantly at P<0.05 (DMRT)

Table 3:Mean effects of Temperature on antagonistic activities of potential antagonists on a pathogen in dual culture on M9 medium

Temp °C	* % Inhibition
15	43.3b
20	64.6c
25	68.1c
30	68.6c
35	35.9a

*Mean of three replicates after 7 days of incubation
Means in a column followed by different letters differ significantly at P<0.05 (DMRT)

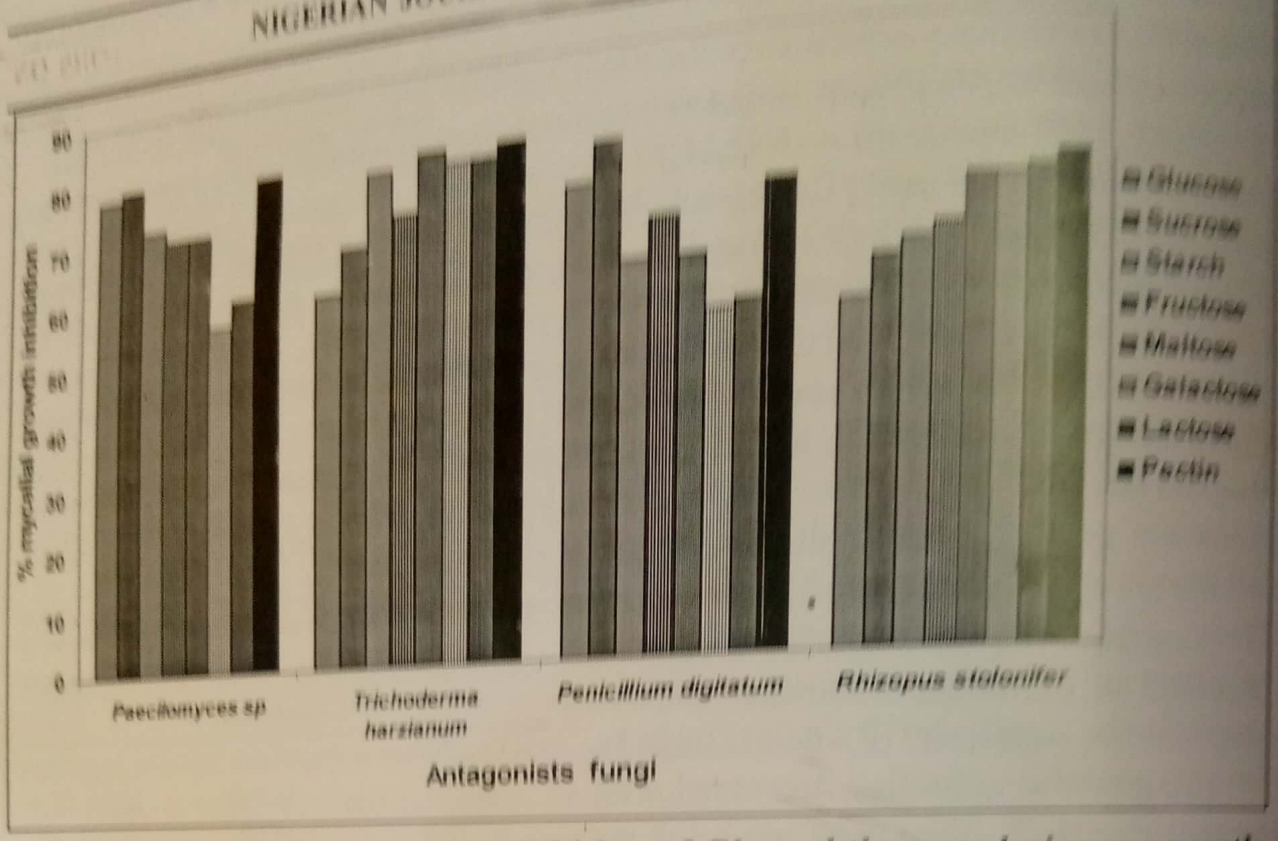

Fig.1. Effect of carbon sources on inhibition of *Phytophthora palmivora* growth in dua culture with antagonists after 7 days of incubation

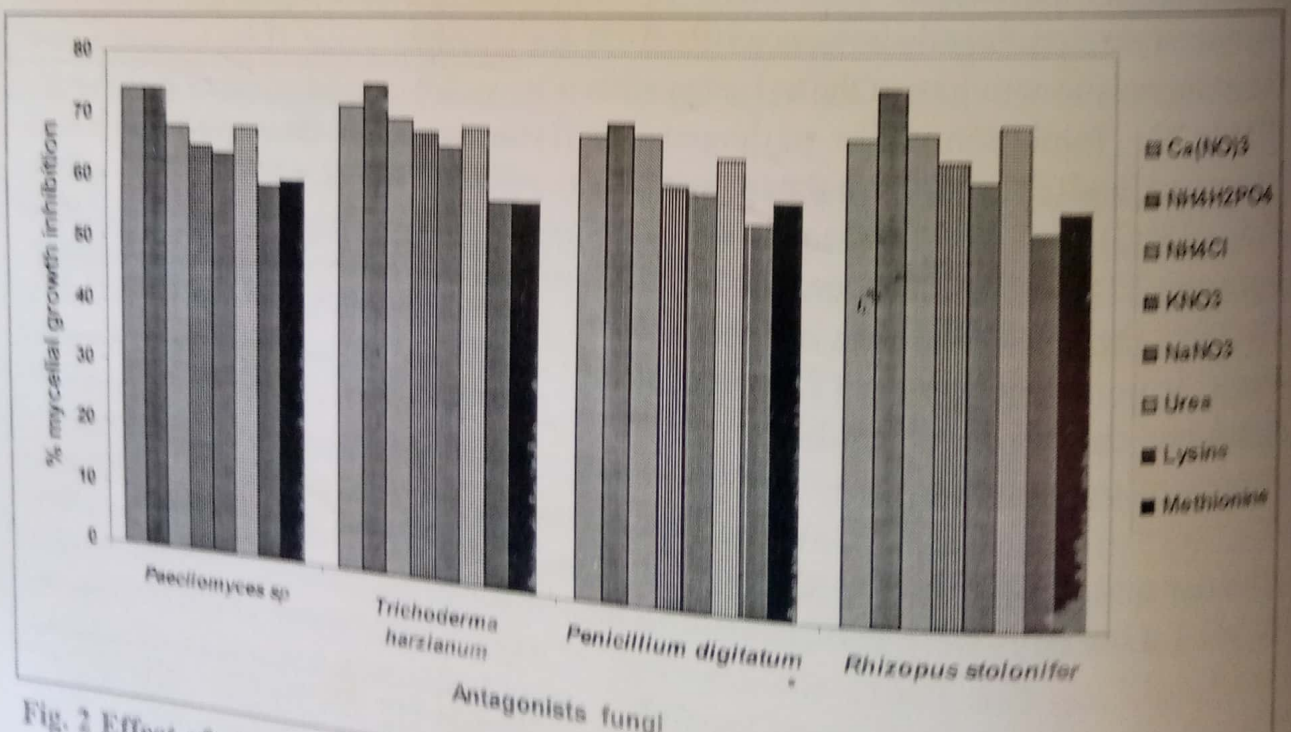

Fig. 2 Effect of nitrogen sources on inhibition of *Phytophthora palmivora* growth in dua culture with antagonists after 7 days of incubation

DISCUSSION

The result showed that all the media tested enhanced the parasitism of *P. palmivora.* These media increased the growth rate of the antagonists and by inference prevented the pathogen from being well established. Thus these substance could be effective in formulating media for biological control of black pod fungus of cocoa. The result on the effect of nutritional factors on antagonistic activities of the test fungi against *P. palmivora* revealed some preference for some ranges of carbon and nitrogen sources. Carbon and nitrogen nutrition may be essential for successful production of inoculum biomass, for sustaining biological activity as well as for the growth of both the pathogen and the antagonist (10,15). It was observed that some nutritional factors significantly affected the antagonistic activities of the potential antagonists against *P. palmivora*. The utilized carbon sources that produced the highest level of antagonism were pectin and glucose, which revealed that the optimal production of the antifungal metabolites by these antagonists require suitable nutritional conditions. High levels of antagonism were also observed in the medium supplemented with nitrogen sources among which are Ammonium chloride, Calcium nitrate, Ammonium hydrogen phosphate and urea. These are common ingredients of fertilizers (7,20).It had been reported by(16) that the Ammonium and Nitrogen fertilizer used in agricultural activities in Western Australian wheat belt has suppressive effect on take all fungus (*Gaeumannomyces graminis*). This research showed that the carbon and nitrogen sources enhanced the antifungal activities of all the test antagonists against *P. palmivora.* The addition of sucrose that might improve the ability of biocontrol agent to colonize witches brooms and promote sporulation in the field was also reported.(9).

The environmental factors were found to greatly influence the antagonistic activities of the test antagonists against *P. palmivora.* The antifungal activity of all the potential antagonists was at optimum between 25 - 30°C. However outside 30°C and below 15°C the antifungal activity dropped. It had been earlier pointed out that the broad temperature range (15 -30°C) for the antifungal activity of sterile red fungus enhanced its potential to be used as a biocontrol agent in the Western Australian wheat field (6). Strong inhibition of pathogen growth was observed to increase with decrease in pH this study. This result is in agreement with the findings of (17) and (21) who separately reported that the optimum pH for inhibitory activity of biocontrol agents was approximately 5. The effect of pH on nutrient utilization might be due to the changes in the bonding potential of various compounds and differential permeability of cell membrane at different pH levels. For example the cells of *Zygorrhynchus moelleri* were reported to be permeable to glucose and acetate only at pH 6.8 whereas at pH 3.4 the cells were permeable to all the tricarboxylic acid cycle intermediate but not to acetate (15). Findings of this research revealed that environmental and nutritional factors are essential for successful

production of inoculum biomass as well as for sustaining biological activities. Further investigations into the efficacy of these potential antagonists at preventing the invasion of cocoa pods by the black pod disease pathogen (*P. palmivora*) are in progress.

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