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Allelopathic potential of some biocontrol agents for the control of fungal rot of yellow yam (*Dioscorea cayenensis* Lam)

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The adverse effect of synthetic pesticides on human health and the natural ecosystem necessitate the need to explore natural mechanisms of disease control in plants. This study evaluated the allelopathic potential of five biocontrol agents: *Trichoderma longibrachiatum*, *Trichoderma asperellum*, *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas fluorescens* in the control of six fungal pathogens associated with tuber rot of *Dioscorea cayenensis*. Rotten tuber samples were randomly collected across three agro-ecological zones (AEZs): humid rainforest (HF), derived savanna (DS), and southern Guinea savanna (SGS) in Nigeria. Biocontrol agents were isolated from the yam rhizosphere using the serial dilution method; the agar pairing method was used for the *in vitro* trials. The destructive sampling method was used to evaluate rot control by the antagonists *in vivo*. *Aspergillus niger* had the highest incidence of 64.71% across the HF, 52.08% across the DS, and 41.98% across the SGS. *B. subtilis* had the highest inhibitory zone of 16.7 ± 0.05% when paired with *A. niger*, 15.4 ± 0.01% with *Lasiodiplodia theobromae,* 14.0 ± 0.33% with *Penicillium oxalicum*, 7.1 ± 0.14% when paired with *Rhizoctonia solani*; 17.1 ± 0.11% with *Sclerotium rolfsii*, and 10.3 ± 0.94% with *Fusarium oxysporum*. All biocontrol agents significantly (P = 0.05) reduced rot development of the test pathogens relative to the control in the *in vivo* experiment. The establishment of a distinct zone of inhibition, especially by the bacterial antagonists attests to the fact that they produced allelochemical substances. Therefore, further research is recommended to evaluate the biochemical composition of these microbial metabolites, their level of toxicity, and fate in the environment.

Key words: Allelopathic potential, biocontrol agents, tubers, allelochemicals.

INTRODUCTION

Yellow yam (*Dioscorea cayenensis*) is an important food crop, especially in the yam zone of West Africa which accounts for 90% of world yam production with Nigeria being the first producer (Osunde, 2008). Babaleye (2003) reported that yam contributes more than 200 dietary calories per capital daily for more than 150 million people

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Plate 1. *Dioscorea cayenensis* tubers attacked by fungal rot disease under natural infection.

in West Africa and serves as an important source of income to the people. Postharvest deterioration of yams has been attributed to insects, nematodes, respiration of the dormant tuber, sprouting, and microbial attack (Ikotun, 1989; Okigbo, 2005). Microbial rot accounts for the highest losses in yam tubers during storage, with fungi being responsible for greater loss than any other single cause (Amusa et al., 2003). The major fungal pathogens associated with storage rot of yams include *Lasiodiplodia theobromae*, *Penicillium oxalicum*, *Aspergillus niger*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium oxysporum* (Green et al*.*, 1995; Dania, 2012).

The use of pesticides in crop protection has been the target of considerable criticism (Manjula et al., 2005). Though chemicals have played a significant role in maximizing productivity, extensive use of broad spectrum compounds has resulted in harmful and undesirable effects on the environment (Adesiyan, 1986). In addition, the appearance of fungicide-resistant fungal pathogens and new exigencies of laws concerning food quality and environmental conservation complete the challenge for alternative disease management strategies in crop protection research (Dayan et al., 1999). Peasant farmers constantly have rot problems to contend with annually in storage of cultivated yams. The drawbacks of chemical control now leave them with natural control options of protecting their harvested yam from spoilage and avoid yield losses.

Therefore, allelopathy has become a viable alternative in natural crop protection (Francisco et al*.,* 2003). It entails biochemical processes in which secondary metabolites from plants and microorganisms are involved, affecting growth and development of biological systems (Ries et al, 1994; Francisco et al*.,* 2003). Allelopathy in microorganisms involves the release of a chemical substance, an allelochemical or antibiotic, into a medium or an environment which acts as a growth inhibitor to another organism. Biological control agents (BCA) are known to produce allelochemical substancces (He et al*.,* 2006). Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites such as harzianic acid, alamethacin, tricholin, viridian, and peptailbols which inhibit the growth of plant pathogens (Vey et al., 2001). Similarly, *Bacillus subtilis* has been reported to produce allelochemicals such as bacillomycin, surfactin (Tsuge et al., 1995), bacitracin, subtenolin, and subtilin (Manjula et al., 2005).

Although several authors have researched extensively on the ability of these BCA to produce secondary metabolites as previously stated, most of these experiments often do not go beyond the *in vitro* trials. This, therefore, leaves an information gap on the applicability of the results *in vivo*. Hence the objective of this study was to determine the potential of the allelochemicals produced by some BCA for the control of fungal pathogens causing rot of *D. cayenensis* tubers in storage.

MATERIALS AND METHODS

Collection of yam samples

Rot-infected tubers of *D. cayenensis* were collected from 14 Local Government Areas (LGA) across three agro-ecological zones (AEZs) in Nigeria: humid rainforest, derived savanna/forest transition, and southern Guinea savanna (plate 1). Three tubers showing symptoms of rot were randomly collected from each of five farmers' barn in each LGA across the AEZ. A total of 210 rotted tuber samples were screened for infecting fungi. Healthy susceptible tubers of genotype TDc 98-172 obtained from the International Institute of Tropical Agriculture (IITA) yam barn, Ibadan were used for *in vivo* trials.

Isolation of test pathogens

Infected tubers were washed with sterile distilled water (SDW) and cut open to expose the fresh necrotic lesion. Tissues measuring 2 × 2 mm were cut from areas in advanced necrosis, surface-sterilized with 10% sodium hypochlorite for 1 min, and rinsed 5 times in SDW. Then, the samples were plated on potato dextrose agar (PDA), incubated at 28 ± 2°C for 2-3 days and examined for fungal growth. The cultures were further purified and pure fungal isolates viewed under a compound microscope and identified using standard procedures (Barnett and Hunter, 1998).

Distribution of fungal isolates among yam species

This was determined in order to ascertain the occurrence of the fungal pathogens across the three AEZ. This was evaluated by the expression of the number of times each organism was isolated from each species per AEZ as a percentage of the total different organisms from all the AEZ. This was calculated thus:

Incidence of isolate = $^{p}/_{Q} \times 100/_{1}$

Where Q= Total number of micro fungi isolated per yam species in

the locations and P= number of times of occurrence of the individual isolate per yam species in the locations.

Quantification of fungal spores

Spore suspension of each fungal isolate was prepared by adding 10 mL of SDW to 7-day-old culture plates to obtain 10 mL spore volume. The resultant solution was scooped with a spreader and then filtered through cheese cloth. Tween 80 (0.5 ml/L) was added to the fungal culture in Petri dishes to enhance removal of their spores. Fructification and spore count were determined using an hemacytometer. Determination of spore concentration was done by dispensing 0.1 mL of inoculum on the hemacytometer. Spores in the four corner squares and at the center were counted and multiplied by 10^4 to determine the total spore count. Final spore count and inoculum concentration were determined using the method of Berkane et al. (2002). Bacterial inoculum concentration was determined by serial dilution using the method of Okechukwu et al. (2000).

Isolation of biocontrol agents

Biocontrol agents used in this study: *Trichoderma longibrachiatum*, *T. asperellum*, *B. subtilis*, *B. cereus*, and *Pseudomonas fluorescens* were isolated from yam rhizosphere at IITA research plots using the method of Okigbo (2005). One gram of the soil sample was dissolved in 10 mL of SDW in a McCartney bottle. The solution was left for 1 h and shaken at 20 min intervals to acclimatize the microorganisms at ambient temperature. Serial dilution was done to obtain 10^6 spores/mL for fungal antagonists and 10^8 cfu/mL for bacterial antagonists. Thereafter, 1 mL of each dilution was dispensed into separate 9-cm Petri dishes. PDA and nutrient agar maintained at 45°C were poured over the suspension for fungal and bacterial antagonists, respectively. However, *P. fluorescens* was cultured on Kings B medium which is selective and encourages the production of fluorescent pigments. A litre of the medium consisted of proteose peptone 20 g, K_2HPO_4 1.5 g, $MgSO_4.7H_2O$ 1.5 g, glycerol 10ml, agar 15 g and distilled water 1000 ml. Inoculated Petri dishes were incubated at 28 ± 2°C for 24-48 h before being observed for the growth of the BCA.

Similarly, isolation of *B. subtilis* followed a definite protocol requiring the addition of supplements to enhance its growth while discouraging other bacteria. A loopful of soil obtained from decayed organic matter was suspended in 1 ml of sterile SDW in a test tube. It was mixed well and heated in a water bath at 80°C for 10 min. Thereafter, an inoculating loop was used to streak a sample of the heat treated soil on to peptone –yeast- dextrose plates (PYD) and incubated at 28 ± 2°C for 24-48 h. One litre of PYD was prepared by adding peptone 2 g, yeast extract 5 g, dextrose 15 g and agar 15 g and sterilized at1.05 kg/cm^2 (121°C) for 15 min. Colonies were examined under a compound microscope and those with clear mottled appearance and bearing endospores were again streaked on to fresh plates for purification. These were further subjected to biochemical tests following standard protocols for identification.

Screening of BCA for allelochemicals

The dual culture method was used to test for *in vitro* antagonism. Fully grown culture of each BCA was inoculated using a 3-mm cork borer at four equidistant points, while the test pathogen was inoculated at the center of the plate in three replicates. The culture plates were incubated at 28 ± 2°C for 4 days. Radial mycelial growth was measured on a daily basis. The *in vivo* trial was conducted in a randomized complete block design with three replicates. Prospective BCA in the *in vitro* experiment were tested against the pathogen isolates on healthy tubers of a susceptible IITA genotype TDc 98-172. Healthy yam tubers measuring 300 mm long and about 50 mm wide were used in this experiment. The yam tubers were washed in running tap water to remove any adhering soil particles and later rinsed in SDW. A 3-mm cork borer was used to bore holes 1 cm deep and 10 cm apart at the proximal, middle, and distal regions on each tuber. Three replicates were inoculated for each pair of organisms. A spore concentration of 10^6 spores/mL and 10^8 cfu/mL was used as the inoculum concentration for each fungus and bacterium respectively. Each antagonist and test pathogen was inoculated separately. Each test fungus was inoculated singly to serve as control. All treatments and control were incubated at IITA yam barn for 6 months.

Measurement of zone of inhibition

After incubation of the agar plates for 7 days *in vitro*, the zone of inhibition was measured using a ruler. The zone of inhibition was determined by measuring the diameter of the outer circle (area of clear edge) minus the diameter of the inner circle (growth of producer strain). Three triplicates for each plate were maintained. Inhibition zones were measured in millimetres and converted to percentage.

Data analysis

All numerical data were statistically analysed using generalized linear model (GLM) of SAS. Means were separated using Least Significant Difference Test (LSD) and standard error was determined where applicable at 5% level of significance.

RESULTS AND DISCUSSION

Distribution of fungal isolates among yam species

The distribution of the six pathogenic fungal isolates evaluated in this study is shown in Table 1. *Aspergillus* was the most predominant pathogen across the three AEZs. This was followed by *L. theobromae*, while *S. rolfsii* had the least incidence. The humid rainforest had the highest overall incidence of fungal isolates, followed by the derived savanna and southern Guinea savanna.

This could be attributed to high relative humidity associated with the humid rainforest zone. This result agrees with the findings of Ikotun (1989) who reported that relative humidity and temperature are important determinants of disease incidence. Rot-causing organisms can also be influenced by soil pH, especially by virtue of the fact that they are soil-borne saprophytes (Coyne, 1999). There was significant difference (P=0.05) in the abundance of *A. niger* and *L. theobromae* across the three AEZ. Similarly, the occurrence of *S. rolfsii* and *F. oxysporum* differed significantly ((P=0.05) across the humid rainforest and derived savanna AEZ.

P. fluorescens and *B. subtilis* completely inhibited further growth of *L. theobromae, F. oxysporum* and *A. niger* at four days after inoculation (Plates 2 to 4). A zone of inhibition was established, especially between the bacterial antagonists and the test pathogens *in vitro* (Table 2). This inhibitory zone could be attributed to the fact that the BCA produced allelochemical substances

Table 1. Distribution (%) of six pathogenic fungal isolates on *D. cayenensis* tuber rot across three AEZ in Nigeria.

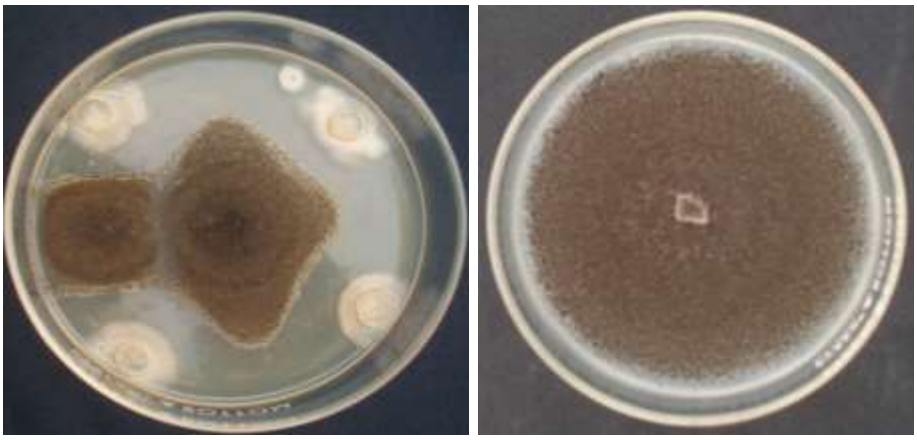
Fungi	Humid rainforest	Derived savanna	Southern Guinea savanna
A. niger	64.71[a]	52.08[a]	41.98[a]
L. theobromae	45.90[b]	29.44[b]	10.11[b]
R. solani	18.22[c]	10.25[d]	4.42[c]
P. oxalicum	5.81[d]	18.09[c]	9.2[b]
S. rolfsii	4.74[d]	2.98[e]	0.0[d]
F. oxysporum	18.92[c]	12.66[d]	10.4[b]

Means with the same letter along the column are not significantly different (P=0.05) using the least significant difference (LSD)

Plate 2. Inhibiting effect of *P. fluorescens* on *L. theobromae*

Plate 3. Inhibiting effect of *B. subtilis* on *L. theobromae*

(antibiotics) *in vitro.* The use of *B. subtilis* and *P. fluorescens* as BCA has been reported (Ries et al., 1994; Okigbo, 2005; Manjula et al., 2005). *B. subtilis* has been reported to produce antibiotics such as bacillomycin, surfactin (Tsuge et al., 1995), bacitracin, subtenolin, and subtilin (Manjula et al., 2005). These antibiotics cause lysis of mycelia of target organisms, which is an indication that lytic enzymes inhibit the growth of pathogens. There was no zone of inhibition between the *Trichoderma* sp. and the test pathogens (plates 5, 6 and 8), except



Treatment A Treatment B (Control)

Plate 4. Inhibiting effect of *B. subtilis* on *A. niger*

Table 2. *In vitro* screening of biological control agents (BCA) for inhibitory zone/allelopathy at 3 days after inoculation (DAI).

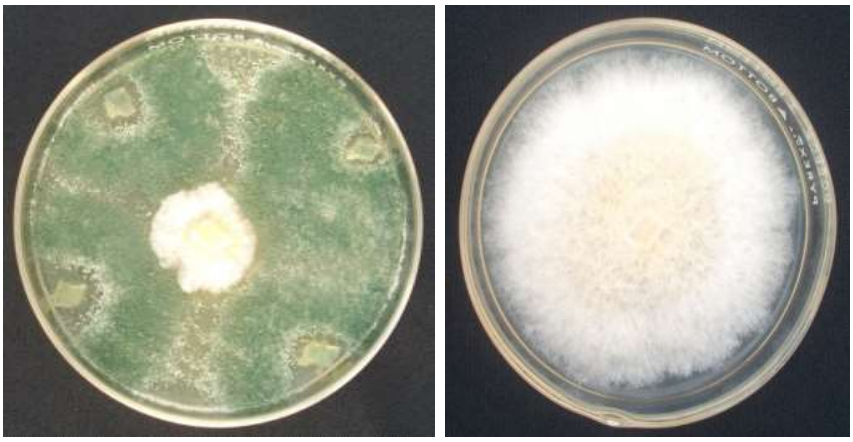
Pathogen	BCA zone of inhibition (%)					
	B. subtilis	***P. fluorescens***	***B. cereus***	**TL**	**TA**	**Control**
A. niger	16.7 ± 0.05	7.4 ± 0.07	10.1 ± 1.2	0.0	7.4±1.08	76 ± 0.09
L. theobromae	15.4 ± 0.01	4.4 ± 0.02	7.8 ± 0.31	0.0	0.0	81 ± 1.44
R. solani	7.1 ± 0.14	2.9 ± 0.7	3.7 ± 0.22	0.0	0.0	77 ± 0.15
P. oxalicum	14.0 ± 0.33	10.2 ± 1.24	9.8 ± 0.04	0.0	0.0	54 ± 1.07
S. rolfsii	17.1 ± 0.11	9.9 ± 2.13	16.7 ± 1.52	0.0	0.0	45 ± 0.22
F. oxysporum	10.3 ± 0.94	7.0 ± 0.01	6.6 ± 0.03	0.0	0.0	37 ± 0.93

TL = *Trichoderma longibrachiatum*; TA = *Trichoderma asperellum.*

Treatment A Treatment B (Control)

Plate 5. Inhibiting effect of *T. asperellum* on *Fusarium oxysporum.*

the inhibitory zone established between *T. asperellum* and *A. niger* (Plate 7). Although there was no zone of inhibition between the *Trichoderma* sp. and the test pathogens, antibiosis was also involved in their control

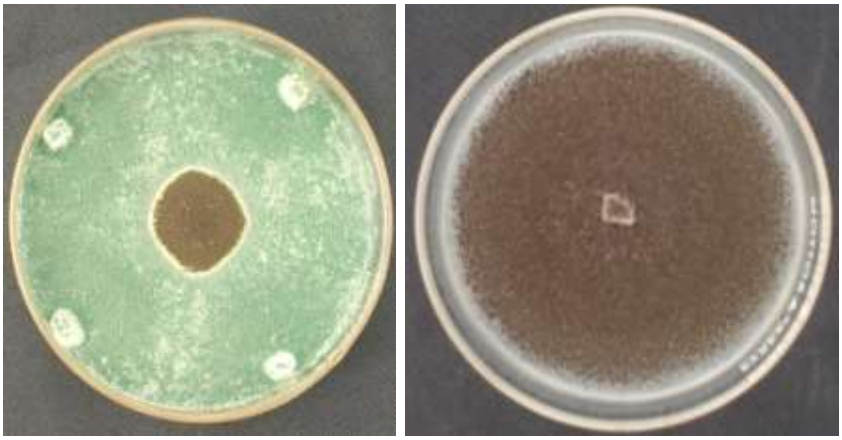


Treatment A Treatment B (Control)

Plate 6. Inhibiting effect of *T. longibrachiatum* on *Fusarium oxysporum.*

Treatment A Treatment B (Control)

Plate 7. Inhibiting effect of *T. asperellum* on *Aspergillus niger.*



Treatment A Treatment B (Control)

Plate 8. Inhibiting effect of *T. longibrachiatum* on *A. niger.*

Table 3. *In vivo* screening (%) of biological control agents (BCA) for tuber rot control at 6 months of storage.

BCA	Pathogen					
	L. theobromae	***R. solani***	***A. niger***	***P. oxalicum***	***S. rolfsii***	***F. oxysporum***
T. longibrachiatum	9.4[a]	10.0[a]	7.2[a]	8.4[a]	9.1[a]	5.8[a]
B. subtilis	5.5[b]	9.4[a]	10.6[b]	8.5[a]	7.4[a]	6.2[a]
P. fluorescens	23.3[c]	19.5[b]	23.9[c]	18.7[b]	24.2[b]	17.7[b]
T. asperelum	8.2[a]	7.1[c]	8.7[a]	7.7[a]	8.8[a]	5.1[a]
B. cereus	4.2[a]	15.5[c]	8.4[a]	15.4[b]	17.1[c]	22.2[c]
Control	56.3[d]	40.2[d]	54.0[d]	40.1[c]	59.9[d]	46.7[d]

Means with the same letter along the column are not significantly different (P=0.05) using the least significant difference (LSD), .

mechanism through the processes of competition and mycoparasitism (Manjula et al., 2005). This was exemplified in the inhibitory zone established between *T. asperellum* and *A. niger*. However, antibiosis in *Trichoderma* species is mostly achieved through direct contact with tissues of pathogens and do not often show inhibitory zone. This result is in consonance with Vey et al. (2001) who reported that *Trichoderma* strains produce volatile toxic metabolites such as harzianic acid, allamethacin, and tricholin. The BCA significantly reduced rot in healthy tubers inoculated with the test pathogens (Table 3). *Trichoderma longibrachiatum* and *T. asperellum* did not differ significantly ($P > 0.05$) in the control of all the pathogens except for *R. solani*.

Interestingly, there was no inhibitory zone between T. longibrachiatum and A. niger, suggesting that the control mechanism may have been mycoparasitism (Plate 8).

Several authors have reported the use of BCA in the control of postharvest plant diseases (Lewis and Papavizas, 1991; Howell, 2003; Manjula et al*.,* 2005). Although the BCA exhibited strong potential for use as biopesticide, there is the need to evaluate their safety in the preservation of ware yam for consumption as well as the environmental impact assessment.

Further research on microbial allelopathy

The inhibition of plant pathogens by antibiotics or chemicals produced by BCA is an allelopathic process and its knowledge is needed to guarantee high yield of crops in any ecosystem. Research on this area will offer a basis for plant growth control and crop improvement. Following the allelopathic potential of BCA used in this study, further research should be focused on the following areas:

(i) Exploration of biochemical processes responsible for their bioactivity.
(ii) Determination of their mode of action.
(iii) Environmental impact assessment of their fate in the ecosystem.
(iv) Evaluation of the toxicity of the allelochemicals so produced.

Provision of solutions to the above questions will lead to starting with the right selection material and extraction method as well as the development of a standard protocol for bioactivity evaluation that will compare between different substances and measure effects over a wide range of target. Allelopathy research transcends isolation, identification, and bioactivity evaluation of a natural product as an allelochemical model. Degradation and stability of allelochemicals in the environment should also be given priority. Therefore, knowledge of the interaction between the allelochemicals produced by BCA and their ultimate degradation in the environment is a key to conservation of a robust ecosystem.

Conflict of interests

The authors did not declare any conflict of interest.

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