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Full Length Research Paper

Biological control of rot-inducing fungi of water yam (*Dioscorea alata*) with *Trichoderma harzianum*, *Pseudomonas syringae* and *Pseudomonas chlororaphis*

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Potential of *Trichoderma harzianum*, *Pseudomonas syringae* and *Pseudomonas chlororaphis* for the control of rot in post harvest yams was investigated. Treatments comprising two pathogenic fungi, each paired with three biological antagonists were set up. Degree of rot was evaluated by calculation of percentage reduction by the antagonists. Pathogenicity test showed that *Botryodiplodia theobromae* and *Fusarium solani* induced rot in healthy yam tubers after fourteen days of inoculation, *B. theobromae* being the more virulent with 85.5% rot. Reduction of rot ranged from 53.5 - 84.5% in *B. theobromae* when paired with the biological antagonists and in *F. solani*, the rot reduction ranged from 59.6 - 87.1%. Generally, *T. harzianum* was the most effective in controlling B. theobromae and *F. solani*. The use of these three biological antagonists, recommends their use in the control of rot in post harvest yam tubers.

Key words: Fungal rot, antagonists, post harvest, yam tubers.

INTRODUCTION

Yams (*Dioscorea sp.*) are the most important food crops in West Africa, East Africa, the Caribbean, South America, India and South East Asia (Coursey, 1967; Okigbo, 2004). FAO (2000) and Okigbo (2002) estimated that the World production of yams is around 20 million tons per year. The greater part of World yam production (over 90%) is derived from West Africa (Coursey, 1967; Okigbo, 2002) and Nigeria alone produces three-quarters of the World total output of yam (Igbeka, 1985; Okigbo, 2002).

Yam tubers are of a very high value, as in food, where it is a major source of carbohydrate, minerals of calcium, phosphorus, iron and vitamins such as riboflavin, thiamine and vitamins B and C (Coursey, 1967; Okigbo and Ogbonnaya, 2006). The principal microorganisms associated with yam include: *Botryodiplodia theobromae* Pat., *Fusarium oxysporum* Schlecht, *Penicillum oxalicum* Currie and Thom, *Aspergillus niger* van Tiegh. and *Aspergillus tamarii* Kita (Adeniji, 1970a; Arene et al., 1985; Okigbo and Ikediugwu, 2000; Okigbo, 2005). Micro organisms that caused rot did so at a high relative humidity and temperatures of 25 - 39°C (Adeniji, 1970a) and some were more aggressive at a higher temperature of 35°C. Yams rots usually start in the soil and progress in storage, which occur when infected tubers do not yet have any sign of external symptoms (Okigbo and Ogbonnaya, 2006).

The use of synthetic chemicals such as borax, captan, thiobendazole, benomyl, bleach (sodium hypochlorite) has been found to significantly reduce storage rot in

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yams (Booth, 1974; Noon, 1978; Okigbo, 2005). The use of micro organisms such as *Trichoderma viride* Pers. ex S. Gray and *Bacillus subtilis* have also be reported (Okigbo and Ikediugwu, 2000; Okigbo, 2002). However, the findings have hardly been adopted by farmers as, in developing economies such as Nigeria, where the majority of farmers can ill-afford the financial cost (Okigbo and Ikediugwu, 2000). Furthermore, chemical pesticides have the additional potential disadvantages of accumulation in the ecosystem and of induction of pesticide resistance in pathogens (Georgopoulos, 1977; Spotts and Cervantes, 1986; Okigbo and Ikediugwu, 2000).

Biological control of plant diseases has proved to be durable in its effects and, has the advantage of not requiring repeated periodic applications as is the case with chemical pesticides. It is thus, potentially better suited for use, particularly in developing economies (Okigbo and Ikediugwu, 2000).

However, the potential of *Trichoderma harzianum*, *Pseudomonas syringae* and *Pseudomonas chlororaphis* for the control of rot in post harvest yams was investigated in the present study.

MATERIALS AND METHODS

Plant materials

Healthy yam (*Dioscorea alata*) tubers of the susceptible variety were obtained from the yam barn of the International Institute of Tropical Agriculture (IITA) and were authenticated by Mr. Ogbe G. (Research Supervisor) of the pathology unit IITA.

Preparation of culture media

Preparation of complete media

Complete media (CM) was autoclaved at 121°C (Cheesbrough, 2000; Jawetz et al., 2004) for 15 min after which it was allowed to cool to 45 - 50°C. 1 ml of 10% concentration of lactic acid solution was added to suppress bacterial contaminants. 9 ml of the molten CM was poured into sterile disposable Petri dishes (6 and 9 cm in diam.) and was allowed to gel.

Preparation of potato dextrose agar (PDA)

Potato dextrose agar was used as the medium for the growth and maintenance of *B. theobromae*, *T. harzianum*, *P. syringae* and *P. chlororaphis* isolates. The PDA was prepared according to the manufacturer's recommendations by dissolving 39 g of dehydrated PDA (Difco™ Becton, Dickinson and Company Sparks, USA) in 1 litre of distilled water and autoclaved at 121°C for 15 min (Cheesbrough, 2000; Jawetz et al., 2004). The media was allowed to cool to 45 - 50°C and 1 ml of 10% concentration of lactic acid solution was added to suppress bacterial contaminations. 9 ml of the molten PDA was then poured into sterile disposable Petri dishes (6 and 9 cm in diameter) and was allowed to gel.

Isolation of fungal pathogens from rotten yam tubers

Small sizes of approximately 2 × 2 mm were cut from yam tubers infected with rot at interphase between the healthy and rotten portions of the tubers. According to Ritichie (1991) they were first surface sterilized by dipping completely in a concentration of 10% sodium hypochlorite solution for 2 min; the sterilized sections to be inoculated was then removed and rinsed in sterile distilled water (SDW). The yam pieces were then placed on sterile paper towels in the Laminar Air Flow Cabinet (Environmental Air Control Inc. USA) to dry for 5 min. Three piece of the yam sections were plated on five bare PDA plates each and incubated at 27°C. The plates were examined daily for the development of fungal growth. Fungi were identified using a compound microscope and identification guides (Sutton, 1980). Test fungi for this study were *B. theobromae* and *Fusarium solani*.

Pathogenicity test

Healthy yam tubers (*Dioscorea alata*) were washed under running tap water to remove soil (dirt). According to Ritichie (1991) surface contaminants were removed by dipping each yam tuber into 10% concentration of sodium hypochlorite for 2 min and rinsed twice in sterile distilled water (SDW). The tubers were then placed on sterile paper towels in the Laminar Air Flow Cabinet (Environmental Air Control, Inc. USA) to dry for 20 min. Spore count was taken (10^6 spores/ml) with a haemocytometer for *F. solani* and broken hyphae count for *B. theobromae*. Cylindrical holes were drilled at the proximal and distal ends of the yam tubers, using a sterile 10 mm cork borer. One millilitre of the spore-suspension and broken hyphae suspensions of each isolate were dispensed into the cylindrical holes for each tuber. The part of the tubers bored out were carefully replaced after inoculation and tightly sealed with paraffin to prevent contamination. Another tuber was inoculated with 1 ml mixture of the two pathogenic fungi and the control was set up by inoculating another tuber with 1 ml of sterile distilled water.

Counting of fungi spores

Two drops of the fungi in solution was pipette and placed on a slide. The slide was placed on the haemocytometer and viewed under the light microscope. Spore count was recorded as 1.02×10^6 spores ml/1 litre of SDW.

Counting of bacterial cells

A spectrophotometer (Ultrospee 3000, 80-2106-20-669 89, Cambridge, England) was used in counting the cells of the bacteria.

1 ml of the SDW was pipette into a curvet and placed in the number 1 position of the spectrophotometer. One millilitre of the bacteria in solution was pipette into another curvet and placed in the number 2 position. The number 2 button was pressed to get the reading of cells count. The concentration of the bacteria in liquid was increased or decreased. This was done until a concentration of 0.06 was attained. 0.06 is equivalent to 10^8. The cell count was 1.0×10^8 cells ml/ 1 litre of SDW.

Inoculating of yam tubers with pathogenic fungi and biological antagonists

Healthy yam tubers (*D. alata*) were first washed under running

Table 1. Percentage occurrence of fungi isolated from rotten yam tubers.

Fungi isolated	Percentage occurrence
B. theobromae	16.3
F. solani	13.4
A. niger	13.2
F. oxysporum	11.0
F. semitectum	8.5
A. flavus	9.8
R. stolonifer	11.6
P. oxalicum	9.8
P. chrysogenum	6.4

Table 2. Pathogenicity test showing the percentage rot caused by pathogenic fungi.

Fungi inoculated	Percentage rot
B. theobromae	85.5
F. solani	80.2

tap water to remove dirt and soil particles, before rinsing in 10% concentration of sodium hypochlorite for 5 min to remove surface contaminants. The yam tubers were placed on paper towels to dry.

Treatments comprising two pathogenic fungi, each paired with three biological antagonists were set up to determine the effect of rot in yam tubers. *B. theobromae* and *F. solani* served as the control, while *T. harzianum, P. syringae* and *P. chlororaphis* served as the antagonist.

Each of the biological antagonists was paired with the pathogenic fungi and the yam tubers were inoculated separately as follows:

1. *B. theobromae* paired with *P. syringae*.
2. *F. solani* paired with *P. syringae*.
3. *B. theobromae* paired with *P. chlororaphis*.
4. *F. solani* paired with *P. chlororaphis*.
5. *B. theobromae* paired with *T. harzianum*.
6. *F. solani* paired with *T. harzianum*.
7. *B. theobromae* paired with water (control).
8. *F. solani* paired with water (control).

For each treatment, one litre of the pathogenic fungi in liquid medium was mixed with one litre of the biological antagonist in liquid medium and stirred for 5 min. A sterile pin bar was used to scratch each yam tuber, starting from the proximal end to the distal end. The injured yam tuber was immersed wholly in the dispersed medium (containing the pathogenic fungi and the biological antagonist) and left for 10 min. The yam tubers were then removed and put separately into a moist white nylon bag. The nylon bags were tied with a rubber band and punctured; this was to allow air for conducive environment for the pathogenic fungi. The nylon bags containing the inoculated yam tubers were placed on benches at room temperature for 42 days. Yam tubers dipped in pathogenic fungi in liquid medium alone served as the control.

The yam tubers were evaluated for rot by cutting transversely at the point of injures. The degree of rot was evaluated by comparing with the controls, and the percentage inhibition of rot by the antagonists over the pathogenic fungi was also evaluated according to the method described by Whipps (1987).

$$\text{Percentage Inhibition} = \frac{R_1 - R_2}{R_1} \times 100\%$$

where R_1 is the furthest radial distance of pathogen in control tubers; R_2 is the furthest radial distance of pathogen in antagonist-incorporated tubers.

The inhibition percentage was determined as a guide in selecting the minimum inhibition concentration (MIC) that will be effective in controlling the rot-causing fungi. Antagonists were also rated for their inhibitory effects using a scale by Sangoyomi (2004):

≤0% inhibition (not effective), > 0 - 20% inhibition (slightly effective), > 20 - 50% inhibition (moderately effective), > 50 - <100% inhibition (effective), 100% inhibition (highly effective).

Experimental design

The experimental design used was randomized complete block design (RCBD) with three replicates. Test of variance was calculated using Analysis of variance (ANOVA) via statistical analysis system (SAS) of version 9.1 and means were separated using least significance difference (LSD) via general linear model (GLM) procedure.

RESULTS

Isolation of pathogenic fungi from rotten yam tubers

Fungi that were consistently isolated from rot infected tissues of yam tubers (from inoculation of the rotten yams) include: *B. theobromae*, *F. solani*, *A. niger*, *F. oxysporum, Fusarium semitectum, Aspergillus flavus*, *Rhizopus stolonifer*, *Penicillum oxalicum* and *Penicillum chrysogenum*. The most frequently occurring was *B. theobromae* with 16.3% occurrence, while the least occurrence was *P. chrysogenum* with 6.4% occurrence (Table 1).

Pathogenicity test

The pathogenicity test showed that *B. theobromae* and *F. solani* induced rot in healthy yam tubers after 14 days of inoculation. *B. theobromae* was the more virulent with 85.5% rot (Table 2).

Effects of biological antagonist (*P. syringae, P. chlororaphis, T. harzianum*) on the growth of pathogenic fungi (*B. theobromae* and *F. sarium solani*)

Comparing the radial growth of pathogenic fungi; *B. theobromae* and *F. solani* was higher in the control than

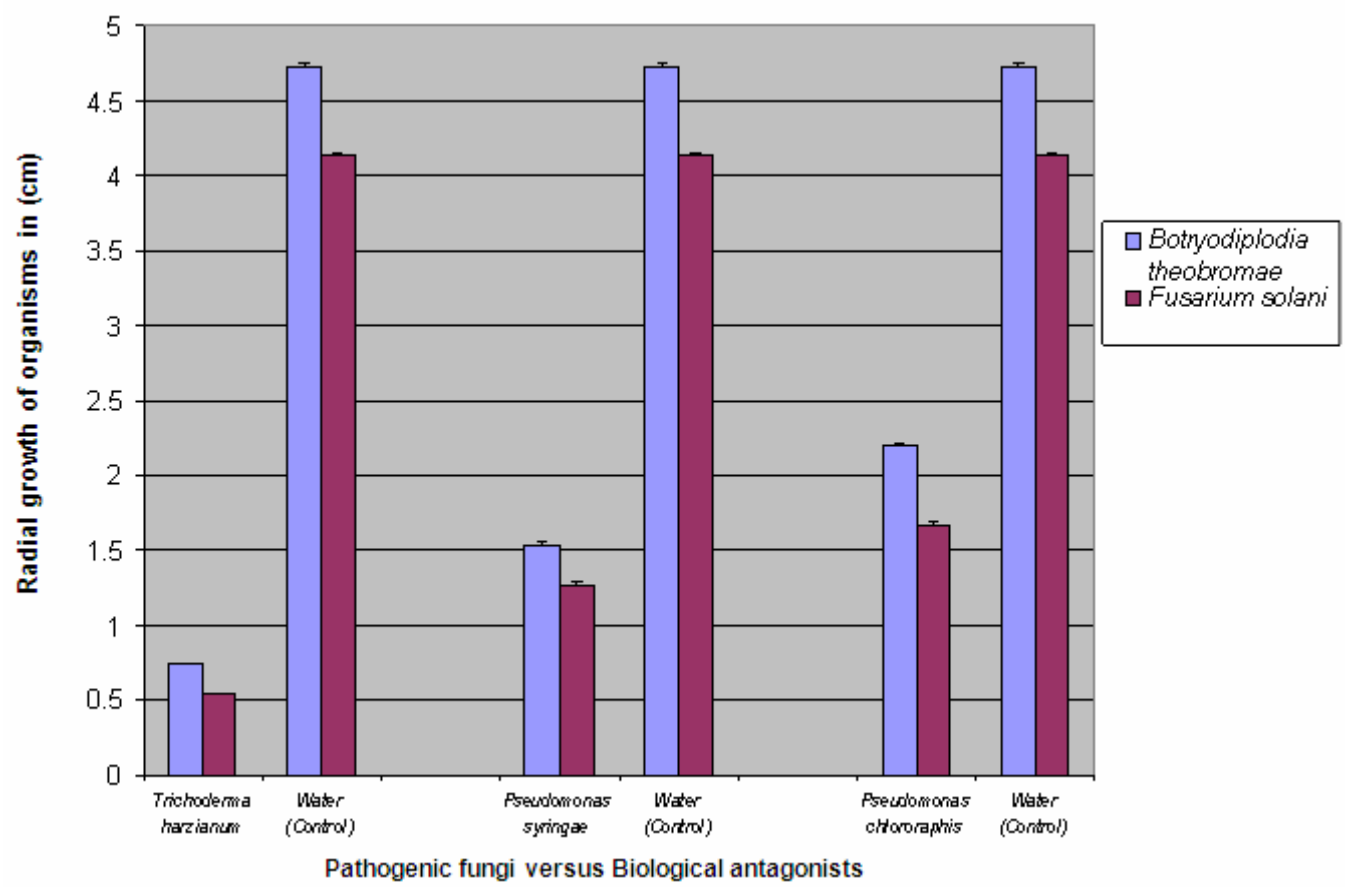

Figure 1. Comparative effect of the three biological antagonists on the two pathogenic fungi.

Table 3. Antagonist effect of *P. syringae, P. chlororaphis* and *T. harzianum* on *B. theobromae* and *F. solani.*

Fungi inoculated	Percentage rot		
	P. syringae	***P. chlororaphis***	***T. harzianum***
B. theobromae	67.6	53.5	84.5
F. solani	69.3	59.6	87.1
Control	0		

when paired with the biological antagonists: *T. harzianum, P. syringae*, and *P. chlororaphis* (Figure 1). The inhibitory effects of the biological antagonist (*P. syringae*) (Table 3) showed that it reduced the growth of *B. theobromae* by 67.6% while that of *F. solani* was reduced by 69.3%. This showed that *P. syringae* was effect on the inhibition of the growth of the pathogenic fungi at ($p < 0.05$). *P. chlororaphis* also showed a reduction in the growth of *B. theobromae* by 53.5% and in *F. solani* the growth was reduced by 59.6% (Table 4). *P. chlororaphis* was effect at ($p < 0.05$) on the growth of the pathogenic fungi. *T. harzianum* showed a reduction in the growth of *B. theobromae* by 84.5% and in *F. solani* by 87.1% (Table 5). *T. harzianum* was effective at ($p < 0.05$) on the growth of the pathogenic fungi.

This study showed that all three biological antagonist were significantly effective ($p < 0.05$) in reducing the rot in the yam tubers treated. Reduction of rot ranged from 53.5 - 84.5% in *B. theobromae* when paired with the three biological antagonists and in *F. solani*, the rot reduction ranged from 59.6 - 87.1% (Tables 3, 4 and 5). This showed a significant difference among the three biological

Table 4. Antagonist effect of *Pseudomonas chlororaphis* on pathogenic fungi (*B. theobromae* and *F. solani*).

Fungi inoculated	Percentage rot
B. theobromae	53.5
F. solani	59.6
Control	0

Table 5. Antagonist effect of *Trichoderma harzianum* on pathogenic fungi (*Botryodiplodia theobromae* and *Fusarium solani*).

Fungi inoculated	Percentage rot
B. theobromae	84.5
F. solani	87.1
Control	0

biological antagonists used in controlling rot caused by *B. theobromae* and *F. solani* in which *T. harzianum* was recorded as the most effective in controlling the pathogenic fungi ($p < 0.05$).

The result of the analysis of the data showed the interaction among the treatments (antagonists versus pathogenic fungi) was highly significant ($p < 0.05$). This indicates that the different pathogenic fungi was highly significantly ($p < 0.05$) affected by the biological antagonists applied and that the biological antagonists differed significantly ($p < 0.05$) from one another with the pathogenic fungi tested.

The mean incidence of rot of *B. theobromae* when paired with the biological antagonists differed from that of *F. solani* significantly ($p < 0.05$) on the treated tubers. Reduction of rot indicates a decrease in the radial growth of the pathogenic fungi. In both test fungi, *T. harzianum* reduced the rot of treated yam tubers (Figure 1) significantly ($p < 0.05$) more than the other biological antagonists.

DISCUSSION

The organisms associated with the rot of *D. alata* in the present study are *B. theobromae* and *F. solani*. These organisms have been associated with post harvest rot as reported by (Ogundana et al., 1970; Okigbo, 2002, 2005; Okigbo and Ogbonnaya, 2006).

The pathogenicity test showed that the pathogenic fungi inoculated in the yam tubers caused rot; this was due to the ability of the pathogen to utilize the nutrient of yam as a substrate for growth and development. This result is similar to the report on fungi associated with Nigerian yams by (Adeniji, 1970; Ogundana et al., 1970; Okigbo, 2000).

The fungitoxic activity of some micro organisms like *B. subtilis*, *T. viride* in controlling pathogens of yam has been reported by Okigbo and Ikediugwu (2000) and Okigbo (2002). Some other biological control measures like plant extracts have been used to control pathogens of yam (Akueshi et al., 2002; Sangoyomi, 2004; Okigbo and Ogbonnaya, 2006; Okigbo et al., 2009).

In this study, micro organisms- *T. harzianum,* P. *syringae* and *P. chlororaphis* were used to control the pathogens of *D. alata* and these produced a significant inhibition on the growth of the pathogenic fungi on post harvest yam.

T. harzianum may have acted by the production of antibiotic substances that inhibited the growth of *B. theobromae* and *F. solani*; this has been reported by Dennis and Webster (1971a, b) on the production of both non-volatile and volatile antibiotics by species of *Trichoderma*. These substances produced by *T. harzianum* served in the biological control of storage rot of yam tubers, this is seen in the works of Harman et al. (1980), Chet and Baker (1981), Wilson (1989) and Okigbo and Ikediugwu (2000) where species of *Trichoderma* have been exploited in the control of rot fruit and vegetable diseases.

Several works have been reported on the use of bacteria to inhibit the growth of fungi pathogens of yam tubers, as seen in the work of Okigbo (2005) in which *B. subtilis* was used to control post harvest fungal rot of yams. *P. syringae* and *P. chlororaphis* was used in this study to control pathogenic fungi that causes rot in yam tubers. *P. syringae* and *P. chlororaphis* displaced the fungi significantly.

In this study, there was an inhibition of the pathogenic fungi when paired with the biological antagonists: *T. harzianum*, *P. syringae,* and *P. chlororaphis* which is attributed to the displacement of the pathogenic fungi on yam tuber by causing a reduction in the percentage rot observed, showing that the three biological antagonists were effective in the treated tubers. This is also reported by Okigbo (2002) in which *B. subtilis* caused a reduction percentage of rot in treated tubers. This suggest that biological control was in operation and that *T. harzianum*, *P. syringae* and *P. chlororaphis* acted by either producing antifungal substances or colonizing the microsites faster than naturally occurring surface pathogens.

The result of this work has shown that *T. harzianum, P. syringae* and *P. chlororaphis* have potentials to control rot in post harvest yams. This can provide alternative ways in reducing rot in yams than in the use of chemical fungicides. These biological antagonists are less expensive and environmental friendly; an advantage over chemical (synthetic) fungicides.

Further research should be done to step up the development of biological agents in controlling pathogenic fungi. To implement this, the organism should be able to thrive in a conducive environment.

Further work can be carried out by testing these biological antagonists on other fungi, pathogenic to other tuber crops.

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