Journal of
Bioresearch Communications

Gwa and Abdulkadir, J Biores Commun 2017, 1:1

Research Article OMICS International

Biological Control Using *Trichoderma harzianum* against *Penicillium purpurogenum*, Causal Agent of White Yam Tuber (*Dioscorea rotundata Poir*) Rb

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Receive date: July 13, 2017; **Accepted date:** July 26, 2017; **Published date:** August 14, 2017

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Abstract

In vitro assessment of biological control of *Trichoderma harzianum* against *Penicillium purpurogenum* isolated from rotted yam tubers (*Dioscorea rotundata* Poir) in storage was conducted in the Advanced Plant Pathology Laboratory, Federal University of Agriculture, Makurdi, Nigeria. The antagonist and the pathogen were paired in dual culture at different times (antagonist same time with the pathogen, 2 days before the inoculation of the pathogen and 2 days after the inoculation of the pathogen). The inhibitory effect on the growing mycelial of the pathogen on potato dextrose agar (PDA) was measured for a period of 192 hours. Measurement of mycelia radial growth and the percentage growth inhibition (PGI) were done starting from the 72nd hour after incubation. The results of the interactions of the dual culture method showed that both the antagonist and the pathogen mycelial increased with increase in incubation period. Mean percentage growth inhibition (PGI) also increased with the highest inhibition of 69.01% recorded as a result of introducing the biological control agent two days before inoculation of *P. purpurogenum* (2dbipath). The next was by introduction of the bioagent same time with the pathogenic organism (Th×Path) with mean of 38.57%.

The least average inhibition of 22.79% was recorded as a result of introducing the bioagent two days after the inoculation of the pathogen (2daipath). Result of the dual culture method between *T. harzianum* and *P. purpurogenum* differed significantly (P ≤ 0.05) at the different times of assessment. The minimum inhibition concentration (MIC) of *T. harzianum* that was introduced two days before the inoculation of *P. purpurogenum* was the most effective and was therefore considered best for biological management of white yam tuber fungi isolated from rotted yam in storage.

Keywords: *In vitro* assessment; Biological control; *Penicillium purpurogenum*; Yam; Inhibition

Introduction

Yams are important staple food crops in humid and sub-humid tropics [1]. It is estimated that over 90% of world yam production is from West Africa out of which Nigeria produced the largest volume of 35.02 million metric tonnes [2]. Yams are rich in major food nutrients which include carbohydrate, minerals and vitamins [3]. Yam also plays a major role in the socio-cultural significance of the people mostly the South Eastern Nigerian [4] and also in the middle belt of Nigeria among the Tiv tribe [5]. Findings in different parts of Nigeria have demonstrated that microbial organisms are mostly associated with rot of yam tubers in storage [6,7]. Olurinola et al. [8] showed that about 40% of postharvest losses in yam are linked with pathogenic organisms while FAO [9] estimated the loss to be between 50% and 56% respectively after 6 months storage in the yam barn. Pathogenic rots of yam tubers and setts is attributed to fungi organisms such as *Aspergillus flavus, A. fumigatus, A. niger, Botryodiplodia theobromae, Candida albicans, Collectotrichum gloeosporiodes, Penicillium marnessei, Rhizopus stolonifer* [10-13]. The causal agents besides reducing the quantity of yam produced, also reduce the quality of the yam tuber by making them look unpleasant to buyers [14]. Synthetic chemicals such as mancozeb and borax have been found to inhibit growth of rot organisms of yam in storage [15,16]. Application of chemical fungicides to control post-harvest rot causing organisms have been challenged due to the adverse effects on the environment as well as on food and human health, accumulation in the ecosystem and of induction of pesticide resistance in pathogens [17,18]. The most acceptable method of plant disease management is biological control [19,20]. Biological control agents are selective, cheap, produce no resistance to target organisms and are self-propagating and self-perpetuating. The aim of this work was therefore, to study the biological potential of *T. harzianum* on the *in-vitro* control of *P. purpurogenum* associated with rot of yam tubers in storage.

Materials and Methods

Study area

The experiment was performed at the Advanced Plant Pathology Laboratory, Federal University of Agriculture, Makurdi, Nigeria.

Source of *T. harzianum* isolate

T. harzianum used in this study as an antagonist was collected from yam Pathology Unit of University of Ibadan, Oyo State, Nigeria. Stock

cultures of the isolate were prepared and maintained on slants of acidified potato dextrose agar (PDA) in McCartney bottles.

Source of rotted yam tubers

Decayed tubers of Ogoja white yam varieties (*D. rotundata*) with symptoms of rots were collected from different farm barns at different locations in Zaki-Biam market, Benue State of Nigeria. The location lies between longitudes 9˚ 25' and 9˚ 28'E, and latitude 7˚ 32′ and 70 35′N respectively. Deteriorated yam tubers collected were safely protected by keeping them in sterilized polyethylene bags for subsequent isolation and identification of rot causing organisms. Rotted yam tubers brought to the laboratory were protected from rodent attack using wire mesh [21]. The medium used to isolate *P. purpurogenum* as test organism was Potato Dextrose Agar (PDA).

Isolation and identification of *P. purpurogenum*

Pieces of rotted yam tubers measuring 2 × 2 mm were cut out with sterile scalpel at inter-phase between the healthy and rotten portions of the tubers. The tissue sections were dipped in concentration of 5% sodium hypochlorite solution for 2 minutes for surface sterilization; the sterilized sections to be inoculated were then removed and were rinsed four times in Sterile Distilled Water (SDW) [22]. The tissue sections were placed on filter papers in the laminar air flow cabinet for 2 minutes to dry.

Inoculation of *P. purpurogenum*

The pieces of the rotten yam were aseptically transferred onto solidified agar medium in Petri dishes up to five pieces of the infected yam sections were inoculated on three PDA plates each. The plates were incubated for 192 hours at ambient room temperature (30 ± 5°C). Plates incubated were examined at 24 hours interval for fungal growth.

Identification of *P. purpurogenum*

Fungi that grew from the rotted yam pieces were sub-cultured and incubated on separate plates containing sterile acidified potato dextrose agar in order to get pure culture of the pathogenic organism. Morphological characteristics as well as identification of the pure cultures were made and compared with already established standard [23,24].

Test of pathogenicity

Pathogenicity test was carried out according to the method of Amienyo and Ataga, (2006) [25] with some little modifications. The good looking yam tubers were washed with clean tap water for 5 minutes and the tubers were sterilized in 5% sodium hypochlorite solution for 2 minutes. Tubers were cleaned by washing in three successive changes of sterile distilled water and were later dried in laminar air flow cabinet for 20 minutes. Cylindrical holes were created using sterilized cork borer of about 5 mm in diameter; a hole of 4 mm deep was made. A disc of 5 mm from a 5 day old pure culture of *P. purpurogenum* mycelial on potato dextrose agar was removed using a sterile cork borer of 5 mm in diameter and was placed in each hole respectively. Holes were completely sealed with petroleum jelly to prevent pathogenic invasion. The procedure was repeated for the control except that potato dextrose agar that was not inoculated with the fungus mycelial was inserted in the holes that were made in the yam tubers. The yam tubers were incubated at ambient room temperature (30 ± 5°C) for 14 days after which the tuber section inoculated were assessed for tissue infectivity by cutting transversely where the yam tubers were inoculated.

Evaluation of antagonistic activities of *T. harzianum in vitro* against *P. Purpurogenum*

Antagonistic activities of *T. harzianum* were evaluated using dual culture method on potato dextrose agar plates. [26] 5 mm diameter mycelial plugs of 5 day old fungal antagonist and pathogen were placed side by side on same Petri dish about 6 cm from each other. The antagonist and the pathogen were plated at three different times (antagonist was plated same time with the test pathogen, two days before the inoculation of the pathogen and two days after the inoculation of the pathogen). The dual and alone cultures were incubated for 192 hours at ambient room temperature (30 ± 5°C). Dishes that were only inoculated with test pathogens were used as controls. Measurement of mycelia radial growths of both the dual culture and the alone culture were carried out at 24 hour interval starting from the 72nd hour till the 192nd hour of incubation. The inhibition of the pathogen was determined according to the method of Korsten and De Jager [27].

PGI (%) =R − R1/R × 100

Where,

PGI=Percent Growth Inhibition

R=the distance (measured in mm) from the point of inoculation to the colony margin in control plate,

R1=the distance of fungal growth from the point of inoculation to the colony margin in treated plate in the direction of the antagonist.

Antagonist was also rated for inhibitory effects using a scale by Sangoyomi [28] as:

≤ 0% inhibition (not effective)

>0-20% inhibition (slightly effective)

>20-50% inhibition (moderately effective)

>50-<100% inhibition (effective)

100% inhibition (highly effective)

Data Analysis

The experimental design used was Completely Randomized Design (CRD) which was replicated three times according to Gomez and Gomez [29]. Analysis of Variance (ANOVA) and statistical F-tests were evaluated at P ≤ 0.05. Differences among treatment means for each measured parameter were further separated using fishers Least Significance Difference (LSD) Cochran and Cox [30].

Results

Isolation of *Penicillium purpurogenum*

P. purpurogenum was isolated and identified as a rot causing fungus of yam tubers in storage. The colony characteristics of the pathogen on potato dextrose agar were found to produce dark bluish color with red pigmentation which spread as the fungus mycelia grew (Figure 1A). Microscopic observation showed that the pathogen conidiophores are branched. The conidia are globules resembling glass beads (Figure 1B).

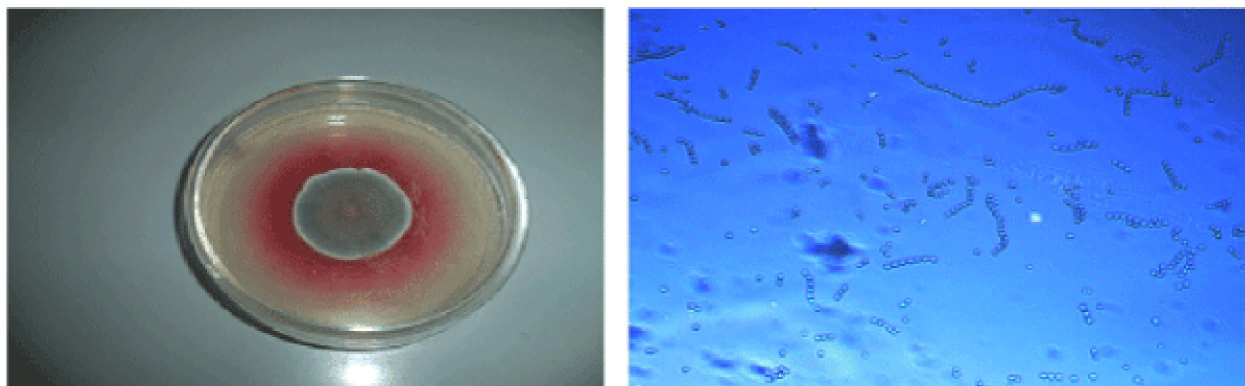

Figure 1: Pure culture of *P. purpurogenum* growing on PDA (left); Microscopic structure of *P. purpurogenum* (X10) (right).

Pathogenicity test

P. purpurogenum isolate was pathogenic on the yam tubers used for the test. Symptoms of rot were observed on the re-inoculated yam tubers as dry brown rot. The control experiments did not show any symptom of infection in the inoculated yam tissue.

Antagonistic effect of the bioagent on inhibition of mycelial of *P. purpurogenum* in culture

The *in vitro* dual culture interactions of the bioagent and the fungus showed significant success in biocontrol of *P. purpurogenum*. It was observed that *T. harzianum* could restrict growth of the pathogens on the culture medium (Figures 2-4). The finding revealed that that the bioagent exhibited antagonistic influence on *P. purpurogenum* in all treatment levels. The bioagent grew faster and higher as the incubation period increased more than the pathogen.

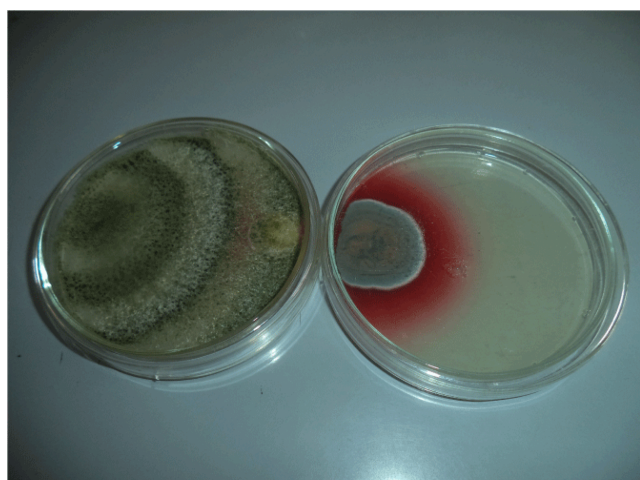

Figure 2: Paired culture of *T. harzianum* and *P. purpurogenum* inoculated same time (Th × path) (left) and pure culture of *P. purpurogenum* as control (right).

The control plates were equally observed to grow more than the fungus in the paired culture. Significant differences ($P<0.05$) in percentage growth inhibition were observed between the pathogen in dual culture with the bioagent. The paired plates showed initial rapid growth of the fungus which stopped at the point of contact with the antagonist (Figures 2-4). The biological antagonist continued to inhibit the mycelial of the fungus and over grew it which resulted in total degradation of *P. purpurogenum* mycelial and sporulation of *T. harzianum* on the entire surfaces of the dual culture plates in all the treatments.

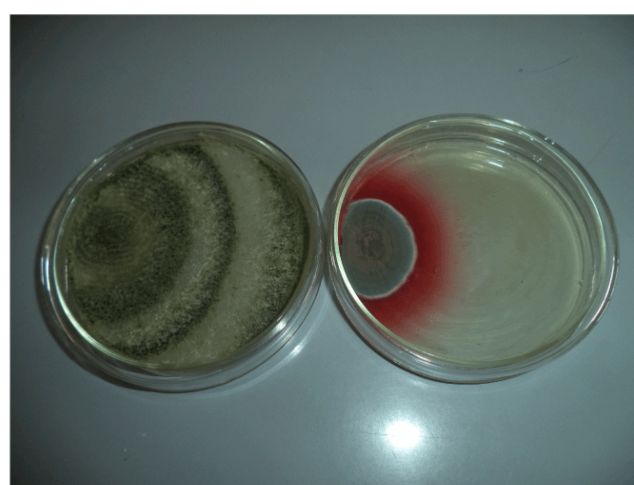

Figure 3: Paired culture of *T. harzianum* and *P. purpurogenum* (left); T. harzianum was introduced 2 days before inoculation of *P. purpurogenum* (2dbipath), P. purpurogenum on potato dextrose agar as control (right).

The inhibition of *P. purpurogenum* when it was inoculated same time with *T. harzianum* rose from 5.81% at 72 hours to 46.72% at 192 hours with a mean percentage growth inhibition of 38.57% while there was slight increase in percentage growth inhibition of the test fungus from 63.89% at 72 hours to 73.15% at 192 hours with a mean of 69.01% when the bioagent was introduced 2 days before inoculation of the fungus. The least mean percentage growth inhibition of 22.79% was got when the bioagent was introduced 2 days after the inoculation of the fungus.

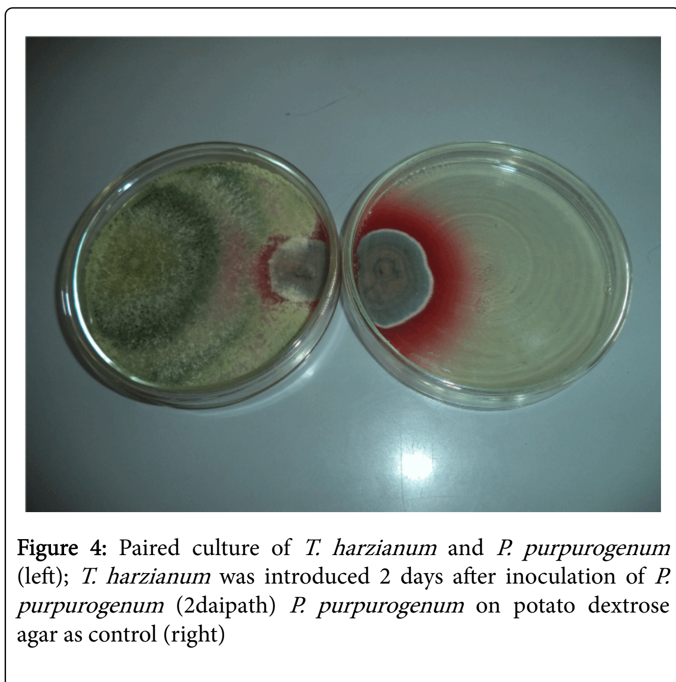

Figure 4: Paired culture of *T. harzianum* and *P. purpurogenum* (left); *T. harzianum* was introduced 2 days after inoculation of *P. purpurogenum* (2daipath) *P. purpurogenum* on potato dextrose agar as control (right)

Dual culture result revealed that *T. harzianum* significantly (P ≤ 0.05) inhibited the growth of *P. purpurogenum* at varying degrees across duration of incubation (Table 1). Mean variation indicated significant differences (P ≤ 0.05) in percentage growth inhibition of *P. purpurogenum* at different times of introduction of *T. harzianum* (Table 1).

Period of Incubation	Time of introduction of *T. harzianum*		
	ThXPath	**Th2dbiPath**	**Th2daiPath**
72 Hrs	5.81 ± 2.91d	63.89 ± 7.35c	8.33 ± 4.81c
96 Hrs	20.82 ± 4.49cd	73.15 ± 3.34a	7.17 ± 0.29c
120 Hrs	30.93 ± 4.61bc	80.98 ± 1.66a	12.78 ± 3.62c
144 Hrs	33.14 ± 8.07abc	85.67 ± 0.59a	22.70 ± 0.83b
168 Hrs	41.67 ± 4.41ab	87.47 ± 0.45a	31.28 ± 2.24a
192 Hrs	46.74 ± 3.79a	73.15 ± 3.34bc	38.83 ± 1.05a
LSD	15.34	10.42	8.26
Mean (LSD=8.97)	38.57 ± 5.16b	69.01 ± 7.09a	22.79 ± 3.22c

Means on the same column with different superscript are statistically significant (P ≤ 0.05); Means on the same row (for Mean) with different superscript are statistically significant (P ≤ 0.05); NB: Th × path=*T. harzianum* introduced same time with pathogen; Th2dbipath=*T. harzianum* introduced 2 days before inoculation of pathogen; Th2daipath=*T. harzianum* introduced 2 days after inoculation of pathogen.

Table 1: Percentage growth inhibitions (PGI) of *P. purpurogenum* at different times of introduction of *T. harzianum.*

Effectiveness of *T. harzianum* in controlling *P. purpurogenum*

T. harzianum was tested at three different levels on *P. purpurogenum* for effectiveness as highly effective, effective, moderately effective, slightly effective and not effective across the treatments. The finding revealed that introduction of *T. harzianum* 2 days before inoculation of *P. purpurogenum* reduced growth significantly (69.01%) better than the introduction of the bioagent same time with the pathogen (38.57%) while the least inhibition (22.79%) was recorded when the antagonist was introduced 2 days after the inoculation of the pathogen. Effectiveness levels of *T. harzianum* were moderately effective to effective and significant (P ≤ 0.05) across treatments (Table 2).

Time of introduction of T. harzianum	Percentage growth inhibition (PGI)	MIC (%)	Level of effectiveness
ThXPath	38.57 ± 5.16b	>20-50	Moderately Effective
Th2dbiPath	69.01 ± 7.09a	>50-<100	Effective
Th2daiPath	22.79 ± 3.22c	>20-100	Moderately Effective
LSD (8.97)			

Th×path=*T. harzianum* introduced same time with pathogen; Th2dbipath=*T. harzianum* introduced 2 days before inoculation of pathogen; Th2daipath=*T. harzianum* introduced 2 days after inoculation of pathogen; MIC=Minimum Inhibition Concentration (%); ≤ 0% inhibition (not effective); >0-20% inhibition (slightly effective); >20-50% inhibition (moderately effective); >50-<100% inhibition (effective); 100% inhibition (highly effective)

Table 2: Effectiveness of *T. harzianum* in controlling *P. purpurogenu.*

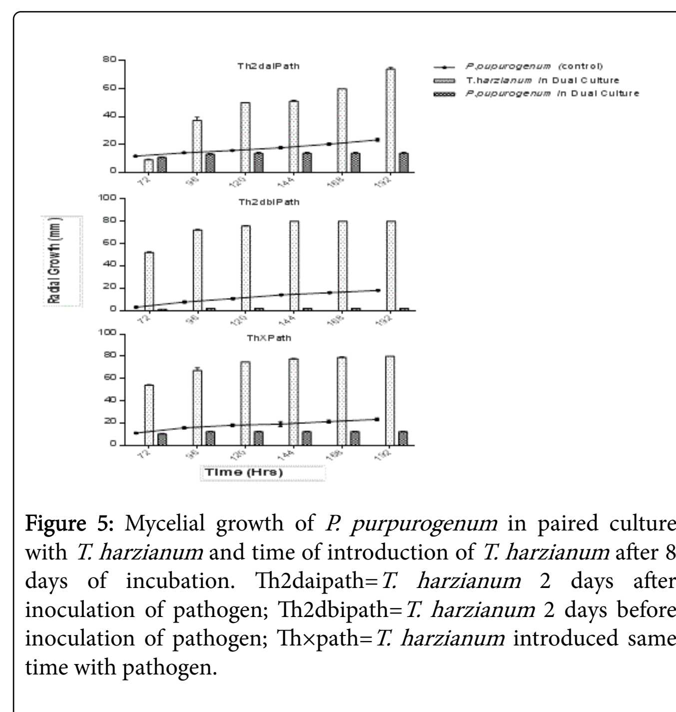

Figure 5: Mycelial growth of *P. purpurogenum* in paired culture with *T. harzianum* and time of introduction of *T. harzianum* after 8 days of incubation. Th2daipath=*T. harzianum* 2 days after inoculation of pathogen; Th2dbipath=*T. harzianum* 2 days before inoculation of pathogen; Th×path=*T. harzianum* introduced same time with pathogen.

Measurement of mycelia radial growth

P. purpurogenum mycelial in the dual culture plates and in the control plates were both measured starting from 72nd hour to 192nd hour. Data collected revealed that the fungus grew in the uninoculated plates more than in the paired culture plates. The antagonist in the dual culture also grew more rapidly than the pathogen and covered the whole plate for each treatment throughout the incubation period (Figure 5).

Discussion

The results of paired culture of *T. harzianum* with *P. purpurogenum* inoculated on potato dextrose agar medium demonstrated that mycelial of *P. purpurogenum* were inhibited by the hyphae of the antagonistic *T. harzianum* when they came when in contact with each other. The mode of action was mainly competition for limited nutrient and space which resulted to starvation and subsequently death of the pathogen [20]. Microscopic and cultural characteristics observed showed mycoparasitic behaviour of the antagonist as it grew and parasitized on the pathogen which caused twisting, air bubbling and disintegration of the pathogen hyphae. Moreover, it may also produce antifungal phenolic compounds [20,32,31]. The finding also showed that *T. harzianum* mycelia entangled the hyphae fragments of *P. purpurogenum* and eventually plasmolysed and lysed them. The conidia of *P. purpurogenum* were torrulose when they came in contact with the mycelial of *T. harzianum* mycelia (Figures 2-4). This report supports the findings of Manjula et al. [33]; Siameto et al. [20]; Mokhtar and Aid [34] as well as Singh and Sharma [35]. Agarwal et al., [36] also reported the antagonistic properties of *Trichoderma sp.* against *A. flavus*, and *A. fumigatus*. Antagonist action using *T. harzianum* against *P. purpurogenum* also confirmed the result reported by Morsy et al. [37]. The inhibition of mycelial growth of *P. purpurogenum* by dual culture could be due to its fast growing nature. Trichoderma species are widely used as biocontrol agents because of their high reproductive capacity, efficient utilization of nutrients, and strong aggressiveness against other pathogenic organisms. The introduction of *T. harzianum* two days before inoculation of the pathogen was in agreement with the work of Campbell [38] who believed that there are no biological control agents that have enough competitive ability to displace a pathogen that has already established itself on the substrate. The success recorded is associated with the time lag between inoculations of *T. harzianum* against *P. Purpurogenum*. This observation confirmed earlier work by Robert [39] and Janisienwicz [40] on the importance of time lag from the arrival of the antagonist and later the pathogen on the phylloplane. According to them there is increase in cell concentration and subsequent colonization of the host by antagonist before the arrival of the pathogenic organism. The results showed that *T. harzianum* grew faster in culture than *P. purpurogenum*. This partly confirmed the report of Adejumo et al. [41] 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 [42] the effectiveness of biocontrol agents might depend partially on their ability to proliferate during as a result of favourable environmental conditions before they encounter plant pathogenic fungi. The Minimum inhibition concentration (MIC) values revealed that the bioagent that was introduced 2 days before the encounter of *P. purpurogenum* inhibited the growth of the fungus more and was therefore considered more effective in controlling the yam tuber pathogen in culture.

Conclusion

The findings of this work strongly suggest that *T. harzianum* can be used for the biological control of yam tuber rot pathogens. This is because the antagonist successfully inhibited the mycelial growth of *P. purpurogenum* at all level of concentrations treated. Also biological control agents are friendlier to environment, target specific, less expensive and biodegradable with no residual effect.

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