



1 ***In vitro* study of antagonistic capability of *Trichoderma harzianum***
 2 **against *Aspergillus niger* isolated from rotten white yam**
 3 **(*Dioscorea rotundata*) tubers**

4
 5 **Abstract:** *In vitro* antagonistic study using dual culture technique was carried out at Advanced Plant
 6 Pathology Laboratory, Federal University of Agriculture, Makurdi, Nigeria to assess the potential
 7 capability of *Trichoderma harzianum* as a biocontrol agent against *Aspergillus niger* isolated from
 8 rotten yam tubers. The test antagonist (*T. harzianum*) was introduced at three different times (same
 9 time with pathogen, two days before the inoculation of the pathogen and two days after the
 10 inoculation of the pathogen). The plates were incubated for 192 hours and measurement of mycelia
 11 radial growths were recorded at intervals of 24hours beginning from the third day. The results of *in*
 12 *vitro* interactions between *T. harzianum* and *A. niger* revealed that *T. harzianum* was able to
 13 significantly ($P \leq 0.05$) inhibit the growth of *A. niger* at the three different times of introduction of *T.*
 14 *harzianum* and this increased with the time of incubation. *T. harzianum* grew faster than *A. niger* and
 15 produced inhibition zones which completely stopped the growth of *A. niger*. Mean percentage growth
 16 inhibition was found to be highest (77.79%) when *T. harzianum* was introduced 2days before
 17 inoculation of *A. niger* followed by introduction of *T. harzianum* same with *A. niger* (45.96%). The
 18 least percentage growth inhibition (28.47%) was recorded when *T. harzianum* was introduced 2days
 19 after inoculation of *A. niger*. In all cases, *T. harzianum* was observed to be effective at checking the
 20 growth of *A. niger* *in vitro* and therefore showed the capability for the biological control of the
 21 pathogen. It is therefore recommended that for effective *in-vitro* control of *A. niger*, *T. harzianum*
 22 should be introduced two days before the arrival of *A.niger*.

23 **Keywords**  *niger*, antagonistic, *in vitro*, *T. harzianum*, yam.

24 **1. INTRODUCTION**

25 Yams (*Dioscorea spp*) are among the oldest recorded food crops and rank second after
 26 cassava in the study of carbohydrates in West Africa [1 and 2]. Yams (*Dioscorea* ) are
 27 reported to be a major staple food crop and source of livelihood for most parts of West
 28 Africa, East Africa, the Caribbean, South America, India and South East Asia [3]. Nigeria is
 29 the largest producer of the crop, producing about 38.92 million metric tonnes annually [4; 5].
 30 Yam has very high food value and is a major source of carbohydrate, minerals such as
 31 calcium, phosphorus, iron and vitamins including riboflavin, thiamine and vitamins B and C
 32 [6; 7]. Rot of yam tubers and setts may be caused by a wide variety of micro-organisms
 33 including fungi, bacteria, and viruses at all stages of growth and also during storage of tubers
 34 [8; 9]. These pathogenic fungi includes *Aspergillus flavus*, *Aspergillus niger*, *Botryodiplodia*
 35 *theobromae*, *Collectotrichum spp*, *Fusarium oxysporum*, *Fusarium solani*, *Geotrichum*
 36 *candidum*, *Penicillium chrysogenum*, *Penicillium digitatum*, *Rhizoctonia spp*, *Penicillium*
 37 *oxalicum*, *Trichoderma viride* and *Rhizopus nodosus* [10; 11; 12; 13]. A total of 30 different
 38 fungi have been reported to be associated with the storage rots of yams [14]. Rot is a major
 39 factor limiting the Post-harvest life of yams besides lack of research for development and
 40 capacity building in yam-based researches [15; 16] and losses can be very high resulting to
 41 about 50% reduction of the total stored tubers reported within the first 6 months of storage
 42 [17]. Losses due to post-harvest rot significantly affect farmers' and traders' income, food
 43 security and seed yams stored for planting. The incidence of rotting varies with the species
 44 and with varieties within each species of yam [18]. [19] reported that rot vary due to
 45 variations in the distributions of the microorganisms and does related to the soil mineral
 46 status because the differences in the mineral status are not known to be correlated with the
 47 type of organism isolated nor total percentage of rot.

48 Several methods have been adopted for controlling losses due to post harvest disease of yam;
49 these include the use of chemicals, use of antagonistic microorganisms, uses of natural plant
50 extracts, as reported by [8]. Because of the low capital income of farmers in Nigeria and lack
51 of expertise in the safe handling of chemical, farmers resorted to the method of crop rotation,
52 fallowing, planting of healthy material and destruction of infected crop cultivars in
53 controlling the diseases of yam tubers [19]. Synthetic chemicals such as borax, captan,
54 thiobendazole, benomyl, bleach (Sodium hypochloride) has been found to significantly
55 reduce storage rot in yams [20; 21; 22] but chemicals have been found to be expensive, can
56 cause environmental pollution and may also induce pathogen resistance. The use of micro-
57 organisms such as *Trichoderma harzianum*, *Trichoderma viride* pers. ex S. Gray, *Penicillium*
58 *digitatum*, *Botryodiplodia theobromae* and *Bacillus subtilis* in the control of fungi pathogens
59 have also been reported [22; 23] but have not been adopted by resource poor farmers in
60 Nigeria. Antagonistic micro-organisms can compete with the pathogen for nutrients, inhibit
61 pathogen multiplication by secreting antibiotics or toxins, or reduce pathogen population
62 through mycoparasitism [24]. It therefore, shows several advantages when compared to
63 chemical products. They decompose more quickly in the environment and are generally less
64 toxic towards non-target species [25]. Fungicides may have a role in the management of yam
65 tuber rots but their cost of application, tolerance of target pathogens and environmental and
66 health concerns may limit application. Thus alternative methods to control post harvest
67 diseases, particularly those that are environmentally safe are urgently needed [26].

68 In view of this, the application of biological control agent (BCA) using *T. harzianum* on the
69 *in vitro* control of *A. niger* causing dry rot of yam tubers in storage therefore, needs to be
70 explored as an alternative to fungicide use.

71 2. MATERIALS AND METHODS

72 2.1 Experimental site

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

75 2.2 Source of *T. harzianum* isolate

76 *T. harzianum* used in this study was obtained from yam Pathology Unit of University of
77 Ibadan, Oyo State, Nigeria. Stock cultures of the isolate were maintained on slants of
78 acidified potato dextrose agar (PDA) in McCartney bottles for subsequent studies.

79 2.3 Collection of diseased yam tubers

80 Rotten yam tubers of white yam varieties (*Dioscorea rotundata*) showing various diseased
81 symptoms of dry rots were obtained from yam farmers in Kadarko, Keana local government
82 area of Nasarawa State, Nigeria which lies between longitude 8° 30' and 8° 35' E, and on
83 latitudes 8° 10' and 8° 14' N. The rotten yam tubers were packaged in sterile polyethylene
84 bags and taken to the laboratory for isolation and identification of pathogens. The tubers were
85 protected using wire mesh to prevent rodent attack [27]. *A. niger* which was the most
86 frequently isolated organism was selected as the test fungus.

87 2.4 Preparation of Potato dextrose agar (PDA)

88 Potato dextrose Agar (PDA) was prepared according to manufacturer's recommendations by
89 dissolving 39g of dehydrated PDA in 1 litre of distilled water and autoclaved at 121°C for
90 15min [28; 29] the medium was allowed to cool to 45-50°C. About 0.16g/l streptomycin
91 sulphate powder was added to suppress bacterial contaminations [30]. 15ml of the molten

92 PDA was poured into sterile 9cm glass Petri dishes and were allowed to cool at room
93 temperature before inoculation.

94 **2.5 Isolation of fungi organism**

95 Small sizes of approximately 2x2mm were cut out with sterile scalpel from yam tubers
96 infected with rot at inter-phase between the healthy and rotten portions of the tubers. They
97 were first surface sterilized by dipping completely in a concentration of 5% sodium
98 hypochlorite solution for 2minutes; the sterilized sections to be inoculated were then removed
99 and rinsed in four successive changes of sterile distilled water (SDW) as reported by [31].
100 The yam pieces were placed on sterile filter papers in the laminar Air flow cabinet to dry for
101 2minutes.

102 **2.6 Inoculation**

103 The bits of the rotten yam were aseptically transferred onto solidified sterile potato dextrose
104 agar (PDA) medium in Petri dishes. Four pieces of the yam sections were plated per plate and
105 each plate was replicated three times and incubated at room temperature ($30 \pm 5^{\circ}\text{C}$) for 8days.
106 The plates were examined daily for the development of fungal growth.

107 **2.7 Characterization and identification**

108 Fungi Isolates were identified after pure cultures were obtained following successive sub-
109 culturing. The culture plates obtained were examined for distinct growth. Microscopic
110 examination and morphological characteristics were noted and compared with existing
111 authorities [32; 30].

112 **2.8 Evaluation of Dual culture method on agar plates**

113 The assay for antagonism was performed on PDA on Petri dishes by the dual culture method
114 [33]. The mycelial plugs (5 mm diameter) of 5 day old fungal antagonist and pathogen were
115 placed on the same dish 6 cm from each other. Isolate of test fungal antagonist was plated
116 same time with pathogen, two days before the pathogen and two days after the pathogen.
117 Paired cultures were incubated at room temperature ($30 \pm 5^{\circ}\text{C}$) for 8days. Dishes inoculated
118 only with the test pathogen served as controls. The experiment was replicated three times in
119 completely randomized [34]

120 **2.9 Radial mycelia growth and determination of inhibition**

121 The radial growths of the pathogen in dual culture and control plates were measured after two
122 days of inoculation at 24 hour interval beginning from the 3rd day up to the 8th day of
123 incubation at ambient temperature ($30 \pm 5^{\circ}\text{C}$). Percent Growth Inhibition (PGI) of pathogen
124 was calculated as described by [35]

$$125 \text{ PGI (\%)} = \frac{R - R_1}{R} \times 100$$

126 Where,

127 PGI = Percent Growth Inhibition

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

130 R_1 = the distance of fungal growth from the point of inoculation to the colony margin in
131 treated plate in the direction of the antagonist.

132 And the width of zone of inhibition (ZI) measured as the smallest distance between the
133 colonies in the dual culture plate if any was determined [36].

134 The percent growth inhibition was determined as a guide in selecting the minimum inhibition
 135 concentration (MIC) that will be effective in controlling the rot-causing fungi for the three
 136 treatments. Antagonist was also rated for inhibitory effects using a scale by [37] as:

- 137 $\leq 0\%$ inhibition (not effective),
 138 $>0-20\%$ inhibition (slightly effective)
 139 $>20-50\%$ inhibition (moderately effective),
 140 $>50-100\%$ inhibition (effective)
 141 100% inhibition (highly effective)

142 *T. harzianum* was tested for both antibiosis and mycoparasitic activities against the test
 143 fungus [24]. The edges of the parasitized pathogen hyphae by microbial antagonist were
 144 transferred from the dual culture dish onto clean slides after 8 days of incubation. Cover slips
 145 were mounted on the mycelia with a drop of lactophenol cotton blue (LCB) [38]. Hyphal
 146 interaction and morphology were examined under a light microscope.

147

148 2.10 Data Analysis

149 Data collected were subjected to Analysis of variance (ANOVA) using GenStat Discovery
 150 Edition 12 for ANOVA and means separation, Minitab Release 14 for descriptive statistics
 151 and Graph Pad Prism 6 for trend graphs. Statistical F-tests were evaluated at $P \leq 0.05$.
 152 Differences among treatment means for each measured parameter were separated using
 153 fishers least significance difference (F-LSD) [39].

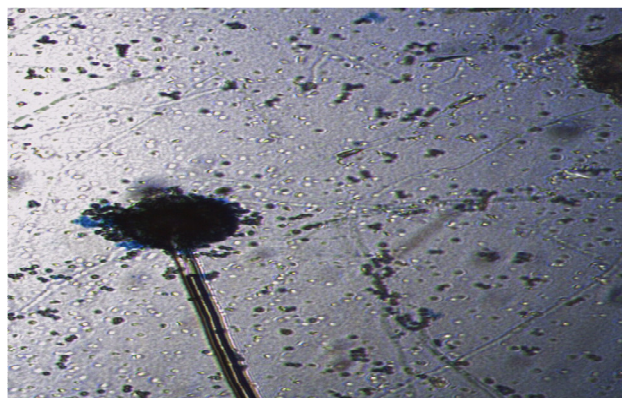
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155 3. RESULTS

156 3.1 Sample collection, isolation and pathogen Identification

157 Test fungus *A. niger* was isolated and identified as one of the fungi causing dry rot of white
 158 yam (*D. rotundata*) tubers in the study area. Macroscopic examination of pure cultures of this
 159 fungus on PDA showed dark brown colour. Microscopic examination and morphological
 160 characteristics and identification showed non-septate conidiophores. Each conidiophores ends
 161 in a terminal enlarged spherical swellings. Conidia are borne by phialides arising from a
 162 terminal swelling on the conidiophores. It has 'mop-like' head of conidia (Fig. 1)

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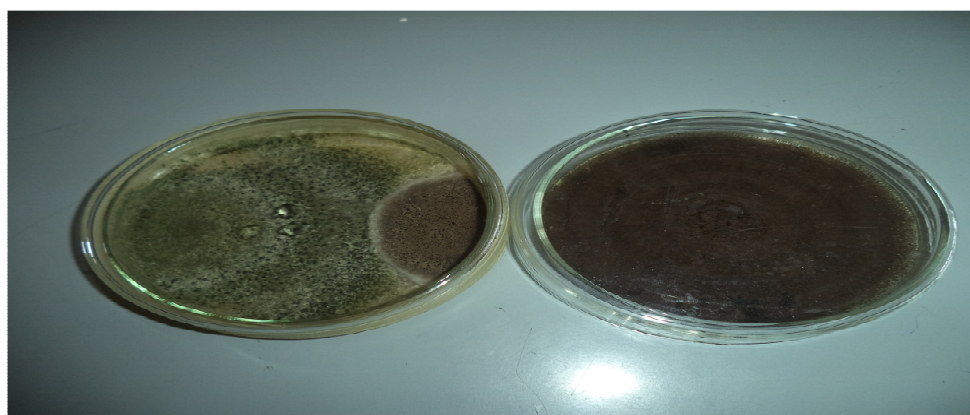


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165 **Figure 1:** Pure culture of *A. niger* growing on Potato dextrose agar (left); Microscopic structure of *A. niger*
 166 ($\times 10$) with conidia borne by phialides on conidiophores (right)

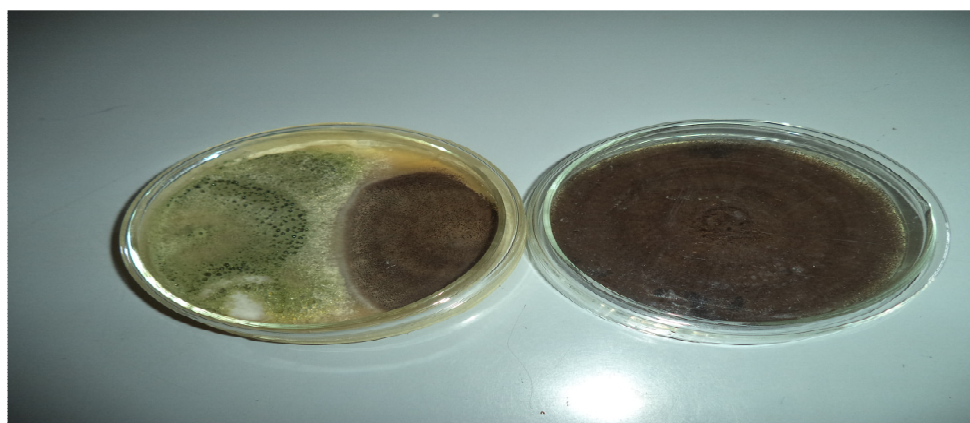
167 3.2 Evaluation of Dual culture method on agar plates

168 The results of dual culture of *T. harzianum* and *A. niger* *in vitro* on PDA medium, shows that
 169 when the mycelium of both cultures came in contact with each other the hyphal growth of the
 170 pathogenic fungus were found to be inhibited by the hyphae of *T. harzianum* (Fig. 2 and 3).
 171 The result of dual culture also shows that the antagonist grew much faster than the pathogen,
 172 parasitized on the pathogen and deprived it from absorbing the nutrients from the substrate.
 173 The pathogen eventually died (Fig. 4). The results of dual culture indicated that *T. harzianum*
 174 significantly ($P \leq 0.05$) inhibited the growth of *A. niger* at varying degrees across duration of
 175 incubation (table 1). When *T. harzianum* was introduced two days before inoculation of *A.*
 176 *niger*, it was observed that the percentage growth inhibition of *A. niger* in dual culture with *T.*
 177 *harzianum* rose steadily from 11.10% at 72 hours to 95.49% at 192 hours after incubation. A
 178 similar trend was recorded when both the antagonist and pathogen were introduced same
 179 time, with percentage growth inhibition of 11.87% at 72 hours to 68.61% at 192 hours
 180 respectively. Inhibitions of 19.39% at 72 hours to 53.40% at 192 hours were computed when
 181 *T. harzianum* was introduced two days after inoculation of *A. niger* (Table 1). It was found
 182 that when *T. harzianum* was introduced two days before inoculation of *A. niger*, the mean
 183 variation in percentage growth inhibition after 192 hours was higher (77.79%) than when
 184 *T. harzianum* was introduced same time with *A. niger* (45.96) and the least percentage growth
 185 inhibition (28.47) was recorded when *T. harzianum* was introduced two days after the
 186 inoculation of *A. niger* (Table 2). Mean variation of percentage growth inhibition of *A. niger*
 187 tested at three different times of introduction of *T. harzianum* significantly ($P \leq 0.05$)
 188 inhibited the growth of *A. niger* (Table 1).



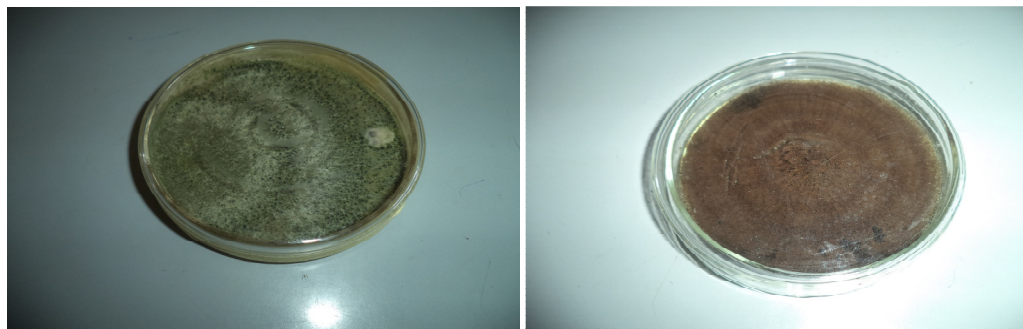
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190 **Figure 2:** Dual culture of *T. harzianum* and *A. niger* on potato dextrose agar inoculated same time
 191 (Thxpath) (left) and pure culture of *A. niger* on potato dextrose agar as control (right)
 192



193

194 **Figure 3:** Dual culture of *T. harzianum* and *A. niger* on potato dextrose agar (left); *T. harzianum*
 195 was introduced 2days after inoculation of *A. niger* (2dai) and pure culture of *A. niger* on potato
 196 dextrose agar as control (right)
 197



198
 199 **Figure 4:** Dual culture of *T. harzianum* and *A. niger* on potato dextrose agar (left); *T. harzianum* was
 200 introduced 2days before inoculation of *A. niger* (2dbi) and pure culture of *A. niger* on potato dextrose
 201 agar as control (right)
 202

203 **Table 1: *In vitro* Percentage Growth Inhibitions (PGI) of *A.niger* by time of**
 204 **Introduction of *T. harzianum***

Duration of Incubation	Time of Introduction of <i>T.harzianum</i>		
	ThXPath	Th2dbiPath	Th2daiPath
72 Hrs	11.87±5.68 ^d	11.10±11.0 ^b	19.39±2.63 ^d
96 Hrs	35.82±4.25 ^c	84.40±2.26 ^a	2.09±1.04 ^e
120 Hrs	44.25±2.56 ^{bc}	89.65±0.21 ^a	19.23±2.63 ^d
144 Hrs	49.95±1.88 ^b	91.81±0.15 ^a	33.13±0.81 ^c
168 Hrs	62.27±1.30 ^a	94.25±0.22 ^a	43.59±0.6 ^b
192 Hrs	68.61±1.16 ^a	95.49±0.35 ^a	53.40±0.94 ^a
LSD	10.03	14.28	5.22
Mean (LSD= 15.88)	45.96±4.58^b	77.79±7.46^a	28.47±4.17^c

205 Means on the same column with the same superscript are not statistically significant ($P \leq 0.05$)

206 Means on the same row (for Mean) with the same superscript are not statistically significant ($P \leq 0.05$) by time
 207 of introduction of *T. harzianum*. Thxpath = *T.harzianum* introduced same time with pathogen; Th2dbipath =
 208 *T.harzianum* introduced 2days before inoculation of pathogen; Th2daipath = *T. harzianum* introduced 2days
 209 after inoculation of pathogen.

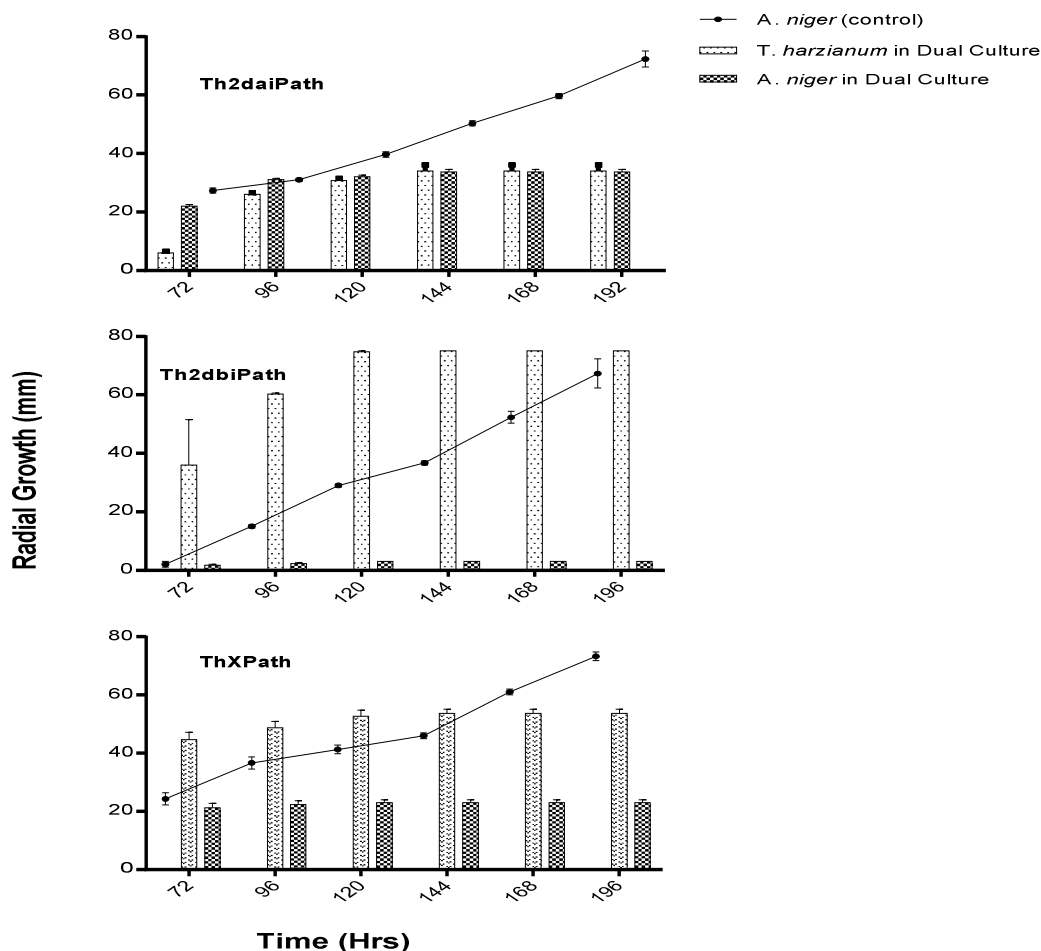
210 3.3 Radial mycelia growth and determination of inhibition

211 Radial mycelia growth of *T. harzianum* and *A. niger* in dual culture and *A. niger* in control
 212 plates for each of the treatments were measured. There was a more rapid growth in the
 213 control plates than in the dual culture plates in all the treatments as observed in Fig.5. It was
 214 also found that *T. harzianum* grew much faster than *A. niger* in all the treatments when grown
 215 in dual culture thereby inhibiting the growth of *A. niger*.

216 Three levels of treatments of *T. harzianum* were tested on *A. niger* for effectiveness levels as
 217 highly effective, effective, moderately effective, slightly effective and not effective across the
 218 treatments. *T. harzianum* introduced 2days before inoculation of *A. niger* significantly ($P \leq$
 219 0.05) reduced growth (77.79%) more than that introduced same time with *A. niger* (45.96%)
 220 and that introduced 2days after inoculation of *A. niger* (28.47%). Effectiveness levels of *T.*

221 *harzianum* were moderately effective to effective and significant ($P \leq 0.05$) across treatments
 222 (Table 2).

223



224

225 **Figure 5:** Radial growth of *Aspergillus niger* in dual culture with *T. harzianum* at different times of
 226 introduction of *T. harzianum* after 196hours of incubation. Th2daipath = *T. harzianum* 2days after inoculation of
 227 pathogen; Th2dbipath = *T. harzianum* 2days before inoculation of pathogen; Thxpath = *T. harzianum* introduced
 228 same time with pathogen.

229 **Table 2: Mean Percentage growth inhibition of *A. niger* treated with *T. harzianum* at**
 230 **different times showing minimum inhibition concentration**

Time of Introduction of <i>T. harzianum</i>	Percentage Growth Inhibition (PGI)	MIC (%)	Level of Effectiveness
ThXPath	45.96±4.58 ^b	>20-50	Moderately Effective
Th2dbiPath	77.79±7.46 ^a	>50<100	Effective
Th2daiPath	28.47±4.17 ^c	>20-50	Moderately effective
LSD	15.88		

231 Th_xpath = *T.harzianum* introduced same time with pathogen; Th_{2db}ipath = *T.harzianum* introduced 2days
 232 before inoculation of pathogen; Th_{2da}ipath = *T. harzianum* introduced 2days after inoculation of pathogen;
 233 MIC = minimum inhibition concentration (%); ≤ 0% inhibition (not effective); >0-20% inhibition (slightly
 234 effective); >20-50% inhibition (moderately effective); >50-<100% inhibition (effective); 100% inhibition
 235 (highly effective)

236

4. DISCUSSION

237 The results of this study revealed that *Trichoderma harzianum* has a high inhibitory effect
 238 against *A. niger* with several biological mechanisms like mycoparasitism and food
 239 competition [40; 41; 24]. The results of dual culture indicated that *T.harzianum* inhibited the
 240 growth of *A.niger* at varying degrees. The bioagent inhibited the growth of the target
 241 organism through its ability to grow much faster than the pathogenic fungi thus competing
 242 efficiently for space and nutrient even as it develops the system of mycotoxins [42]. *T.*
 243 *harzianum* inhibited the growth of the target organisms through its ability to grow much
 244 faster than the pathogenic fungus thus competing efficiently for space and nutrients (Fig. 3, 4
 245 and 5). Mycoparasitism was the most common cause of death for *A. niger* so that
 246 competition for limiting nutrients resulted in biological control of the fungal phytopathogen
 247 [41].

248 The antagonistic effect of *T. harzianum* against *A. niger* *in vitro* on PDA medium showed
 249 that when the mycelium of both cultures came in contact with each other the hyphae growth
 250 of *A. niger* were found to be inhibited by the hyphae of *T. harzianum*. A clear zone of
 251 interaction was formed in all *Trichoderma-A. niger* interactions except in the interaction
 252 between *T. harzianum* and *A. niger*; where *T. harzianum* was introduced 2days before
 253 inoculation of *A. niger* (figure 5). It was observed that *T. harzianum* overgrew *A. niger* and
 254 completely stopped its growth. This is similar to the findings of [43] who showed that *T.*
 255 *harzianum* suppressed the growth of *Pythium aphanidermatum* and *P. myriotylum* killing
 256 their mycelia within three days of inoculation as the test organism were not recovered in the
 257 area grown over by the antagonist. In another experiment, [44] found out that *T. harzianum*
 258 isolates suppressed the growth of *Colletotrichum capsici* eventually overgrowing it within
 259 seven days. The bioagent is known to control plant pathogens by antagonizing them through
 260 mycoparasitism, by producing metabolites such as Beta 1-3 and 1-4 glucanases, directly
 261 competing with the pathogen and inducing host resistance [45; 46]. [47] discovered that the
 262 *Trichoderma sp* isolates have a strong antagonism against wilt diseases caused by *Fusarium*
 263 *sp, in vitro*, on potato dextrose agar medium. It decrease the growth of *Fusarium sp* by
 264 (88%), (86%) and (80%) for *Trichoderma harzianum*, *T.hamatum* and *T. viride* respectively.

265 The inoculation of *T. harzianum* two days before the arrival of *A. niger* was done because
 266 there are no biocontrol agents that have enough competitive ability to displace an already
 267 established pathogen. The time lapse allows adequate increase in cell concentration and
 268 subsequent colonization by antagonist before the arrival of the pathogen [48; 49]. The ability
 269 of antagonists to proliferate within a short period of favourable environmental conditions
 270 before they encounter plant pathogen is an important factor as more rapid growth and
 271 sporulation of fungi from biocontrol formulations may superficially enhance efficacy in the
 272 field. In another case, when *T. harzianum* was introduced 2days after inoculation of *A. niger*,
 273 even when *A. niger* had a significant space and time advantage, *T. harzianum* has shown to
 274 have an antagonistic influence. This effect is produced because of competition for food and
 275 space, mycoparasitism and possible antibiosis [50]. Production of zones of inhibition at the
 276 boundary with the pathogen agrees with the report of [51] that *in vitro* fungal interactions
 277 resulted in production of a zone of inhibition (ZI), contact inhibition or no inhibition at all.
 278 The zones of inhibition produced might be due to the production of antifungal metabolites by

279 the test antagonist as reported by [52] and [53]. Minimum inhibition concentration (MIC)
 280 showed that *T. harzianum* introduced 2days before the arrival of *A. niger* inhibited the growth
 281 of the pathogen at the highest level more than that introduced 2days after the inoculation of
 282 the pathogen as well as that introduced same time. *T. harzianum* introduced 2days before
 283 inoculation of *A. niger* and was therefore considered more effective in controlling the
 284 pathogen.

285 5. CONCLUSION

286 It is therefore, concluded that *T. harzianum* has the capability of affecting the survival and
 287 control of *A. niger in vitro*, one of the pathogens that caused dry rot of yam tubers in storage;
 288 especially when the bioagent is introduced before the arrival of the pathogen on the host.

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