Comparative analysis of inhibitory potential of *Azadirachta indica*, *Trichoderma harzanium* and Copper sulphate on fungal pathogens in *Pleurotus tuber-regium* (Fries) Singer.

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Abstract

In vivo screen-house experiment was conducted in Oyo State College of Agriculture, Igboora to compare the inhibitory potential of Azadirachta indica, Trichoderma harzanium and Copper Sulphate at different concentration of fungal pathogens of Pleurotus tuber-regium. The treatments were laid out in a completely randomized design (CRD) with three replicates. The fruitbodies of P.tuber-regium were inoculated at the base stipe with 160g/l, 240g/l and 320g/l of A.indica, T. harzanium was also inoculated at the concentration levels of 1.0×10^{6}, 1.5×10^{8}, 2.0×10^{10} spores/ml and copper sulphate at 50ppm, 100ppm,150ppm, 200ppm, 250ppm which served at standard while uninoculated served as control. The highest occurrence of pathogenic fungi isolates associated with rots of P.tuber-regium sporophores were Aspergillus flavus (88%), A.niger (85%) and A. ochraceous (84%). The concentration of 2.0×10^{10} spores/ml of T.harzanium gave the highest inhibitory effect on A.niger, A.flavus and A.ochraceous on the cap and stipe lengths (5.60mm and 7.02mm; 5.37mm and 7.18mm; 5.73mm and 10,11mm respectively) of P.tuber-regium. The inhibitory effect of A.indica on the cap length of P.tuber-regium was highly significant against A.ochraceous at 320g/l than other concentration levels and control. $CuSO_4$ as a standard at 250ppm had higher ($p<0.05$) inhibitory effect on the growth of A.ochraceous on the cap and stipe length of P.tuber-regium. Trichoderma harzanium, Azadirachta indica as well as Copper sulphate could be integrated in the management of rots in Pleurotus tuber-regium.

Keywords: *Pleurotus tuber-regium, Azadirachta..*

Introduction

Pleurotus tuber-regium is an edible white rot sclerotia basidiomycetes that belongs to the family pleurotaceac (Alexopolous *et al*., 1996). The fruitbodies and sclerotia of *Pleurotus tuber-regium* have been used traditionally in treatment of anaemia, stomach pain, cough and malnutrition in children (Jonathan and Fasidi, 2003). *Pleurotus tuber-regium* is susceptible to diseases caused by pathogenic bacteria, fungi, viruses, pests which reduce its yield and quality (Olufokunbi; 2011). Several control strategies including fungicides had been adopted but limited information on

suitable environmental friendly biological agents necessitated this study. The antifungal potentials of *Trichoderma harzanium* and *Azadirachta indica* against fungal pathogens had been confirmed on plants but not on *Pleurotus tuber-regium* (Sobowale *et al*., 2007; Anthonia, 2012; Jonathan *et al*., 2012). Therefore the study compared the efficacy of *A.indica*, *T.harzanium* and $CuSO_4$ against fungal pathogens on *Pleurotus tuber-regium.*

Material and methodology

EXPERIMENTAL LOCATION AND DESIGN

In vivo part of the experiment was carried out in the screenhouse of the Department of Agriculture Technology, Oyo State College of Agriculture and Technology, Igboora at 6˚12 north and 2˚24 east of the equator with an annual rainfall of 1700nm and temperature of 28˚C (Sanusi, 2011).

PREPARATION OF SPAWN

In the cultivation of mushroom (*Pleurotus tuber-regium*) in a mushroom farm, the substrate used is a mixture of sawdust, rice bran, lime and water. This was prepared by packing into nylon bags, composted for 30days and pasteurized. The treatment substrate was inoculated with 10g of mushroom spawn (Fasidi *et al*., 2008).

PATHOGENICITY TEST OF THE TARGET PATHOGENS ON MUSHROOM SPOROPHORES.

Pathogenicity test was carried out to make sure that the isolate obtained are pathogenic and able to induce same diseases symptoms on health mushroom plants.

IN VIVO INOCULATION OF BOTANICALS ON *PLEUROTUS TUBER-REGIUM* FRUITBODIES.

The fruitbodies were washed and surface sterilized as described above. The fruitbodies were inoculated with 0.1ml spore suspension (1.0×10^6 spores 1ml) of each of *Aspergillus niger, A. flavus* and *A.ochraceous* and then inoculated with botanical at different concentrations (320g/l, 240g/l and 160g/l W/V) of the plants leaves extracts by extracting the weights (80g, 60g and 40g) of plants materials in 250ml of sterile distilled water simultaneously.

Three replicates were taken for each treatment, incubated at 30^0C for 2 days and observed for the growth rate in the length of pileus and the cap. The control treatment was prepared along with the other treatment. The growth rate of the length of stipe (pileus) and cap of the fruitbodies of *P. tuber-regium* were measured after 2days of incubation with meter rule (Akinbode, 2012).

INOCULATION OF THE *PLEUROTUS TUBER-REGIUM* WITH ANTAGONIST.

Spores of the pathogens and the antagonists were harvested into distilled water separately and stirred continuously into spores suspension. The spore concentration was determined using haemocytometer. The growth effect of the antagonist *T. harzanium* and *T. viride* on the pathogens (*A. niger, A.flavus* and *A.ochraceous*) of *P.tuber-regium* was inoculated with 0.1ml spore of each of the pathogens differently at the stipe base of the fruitbodies using different spore concentration levels of 1.0×10^6, 1.5×10^8 and 2.0×10^{10} for the antagonist according to the injection methods of Dreper and Renfro (1990). Each treatment was replicated thrice with three fruitbodies per replicate. Controls were set up with the inoculation of the fruitbodies with the pathogens alone, antagonist alone and with sterile water. The fruitbodies were observed for development. The length of the pileus (stipe) and length of the cap were measured after 2day of inoculation. The data were recorded and mean values were calculated (Sobowale, 2002).

APPLICATION METHODS OF CHEMICAL TREATMENT

Pleurotus tuber-regium bodies were surface sterile in 10% NaOCL for 1 minutes and then rinsed in changes of sterile distilled water. The fruitbodies were treated with Copper sulphate.

The chemical were used to inoculate the *P. tuber-regium* fruitbodies at the base of the stipe with 0.1ml of different concentration levels from 50ppm, 100ppm, 150ppm,

200ppm and 250ppm, the spore suspensions of the pathogens (*A. niger, A.flavus* and *A. ochraceous*) were also inoculated (1.0×10^6 spores 1ml) into the fruitbodies simultaneously with the chemical. Three replicates were taken for each treatments incubated at 30^0C for 2days and observed for development. The length of the stipe (pileus) and length of the cap of the fruitbodies of mushroom were measured after 2days of incubation. The control experiment was set-up (Jonathan *et al*., 2014)

STATISTICAL ANALYSIS.

Data obtained were analyzed by the Analysis of Variance (ANOVA) using SPSS 20.0 version. The means were separated with Duncan Multiple Range Test (DMRT).

Result

Table 1: The frequency of occurrence of fungal isolate from fruit bodies of *Pleurotus tuber-regium*

Fungi Isolates	Parts of fruit bodies for isolation	Frequency of occurrence
A. niger	Stipe, cap	85.0± 0.3
A.flavus	Stipe cap	88.0± 0.2
A.ochraceous	Stipe cap	84.0± 0.2
Rhizopus spp	cap	25.0± 0.1
Mucor spp	stipe	12.0± 0.1

Table 1: The frequency of occurrence of fungal isolate from fruitbodies of *Pleurotus tuber-regium*

The fungal pathogens isolated from infected fruitbodies of *Pleurotus tuber-regium* were *A. niger*, *A.flavus*, *A.ochraceous*, *Rhizopus* species and *Mucor* species. *A.flavus* had the highest occurrence of (88) followed by *A.niger* (85) and then *A.ochraceous* (84) while the pathogens with lowest frequency of occurrence were recorded for *Rhizopus* spp. (25) and *Mucor* spp. (12). *A. flavus*, *A. niger* and *A. ochraceous* were isolated from the cap and the stipe of *Pleurotus tuber-regium* while *Rhizopus* species was obtained from cap and *Mucor* species were obtained from stipe (Table 1).

Table 2: In vivo assessment of different concentrations of *A. indica* on the cap length and stipe length on *Pleurotus tuber-regium* under inoculation of fungal pathogens.

Concentrations (g/l)	Cap length (mm) A	Stipe length (mm) A
160g/l + *A. niger*	3.02b	6.55c
160g/l + *A. flavus*	3.12b	6.07c

160g/l + *A. ochraceous*	3.18b	7.31ab
240 g/l + A.*niger*	3.52b	6.93bc
240 g/l + *A.flavus*	3.23b	6.57c
240 g/l + *A. ochraceous*	3.36b	7.31ab
320 g/l + *A.niger*	3.18b	7.21ab
320 g/l + *A.flavus*	3.36b	6.57c
320 g/l + *A. ochraceous*	4.36a	7.40a
Control(O)	0.00c	0.00d

*Means of three replicates (mm).

Means in the same column followed by the same letter are not significantly different at P≥ 0.05

Table 2: In vivo assessment of different concentrations of *A. indica* on the cap length and stipe length on *Pleurotus tuber-regium* under inoculation of fungal pathogens.

The inhibitory effect of *A. indica* on the cap length of *P. tuber-regium* was highly significant against *A.ochraceous* at 320g/l than other concentration levels and control. There was no significant difference among other treatment and control for *A.indica* (Table 2).

Table 3: In vivo assessment of *T. harzanium* against fungal pathogens on *Pleurotus tuber-regium.*

Mushroom bags *(Pleurotus tuber-regium;* Concentration(spores/ml)		Cap length (mm) *T.harzanium*	Stipe length (mm) *T. harzanium*
A.flavus	$1.0x10^6$	4.01d	7.82b
A.niger		4.24d	7.71b
A.ochraceous		4.69bcd	8.26b
A.flavus	$1.5 x10^8$	4.74bcd	7.48b
A.niger		4.69bcd	7.46b
A.ochraceous		5.13abc	7.54a
A.flavus		5.37ab	7.18b

A.niger	$2.0x10^{10}$	5.60a	7.02b
A.ochraceous		5.73a	10.11a
Control		4.28d	4.60d

Means in the same column followed by the same letter are not significantly different at P≥ 0.05

Table 3: In vivo assessment of *T. harzanium* against fungal pathogens on *Pleurotus tuber-regium.*

The inhibitory effect of *T.harzanium* on the length of *P.tuber-regium* is significantly higher ($p<0.05$) against *A.ochraceous* at 2.0×10^{10} spore concentration level compared with other concentration levels of fungal spores and control. There was no significant difference for 1.5×10^{8} *T.harzanium* against *A.flavus* and A.niger and 1.0×10^{6}*T.harzanium* against *A.ochraceous* on the cap length of *P.tuber-regium* as well as 1.0×10^{6}*T.harzanium* against *A.flavus* and *A.niger* (Table 3).

Table 4: In vivo assessment of $CuSO_4$ at different concentration on cap length and stipe length on *Pleurotus tuber-regium* (standard).

Concentration (ppm)_	Cap length(mm) $CuSO_4$	Stipe length(mm) $CuSO_4$
50 ppm + *A.niger*	2.13e	2.50i
50 pzpm + *A. flavus*	2.30de	3.40hi
50 ppm + *A. ochraceous*	2.37de	3.37hi
100 ppm + *A. niger*	2.40de	4.50gh
100 ppm +*A. flavus*	2.67de	4.37ghj
100ppm + *A. ochraceous*	2.57de	3.63def
150 ppm + *A. niger*	2.43de	4.63gh
150 ppm + *A. flavus*	3.17cde	5.27fg
150ppm + *A. ochraceous*	3.27cde	5.13fg
200 ppm + *A. niger*	3.17cde	7.27de
200 ppm + *A.flavus*	3.33cde	7.07de
200 ppm + *A. ochraceous*	3.40cde	6.40ef
250 ppm + *A.niger*	4.40bc	8.67bc

250 ppm + *A. flavus*	4.47bc	8.57bc
250 ppm + A. ochraceous	6.43a	9.50a
Control *(A. niger*)	0.00f	0.00k
(*A. flavus*)	0.00f	0.00k
(A. ochraceous)	0.00f	0.00k

*Means of three replicates (mm).

Means in the same column followed by the same letter are not significantly different at P≥ 0.05

Table 4: In vivo assessment of $CuSO_4$ at different concentration on cap length and stipe length on *Pleurotus tuber-regium* (standard).

Also, there was no significant difference among the different spores concentration levels of *T.harzanium* against the isolated fungi except for 1.5×10^8 and 2.0×10^{10} spores/ml against *A.ochraceous* on the stipe length of *P.tuber-regium*. Moreover, $CuSO_4$ as standard, the concentration of $CuSO_4$ at 250ppm had higher ($p<0.05$) inhibitory effect on the growth of *A.ochraceous* on the cap and stipe length of *P.tuber-regium* (Table 4). There was no significant difference for 150ppm $CuSO_4$ against *A.niger*, *A.flavus* and *A.ochraceous* as well as 200ppm $CuSO_4$ on *A.niger*, *A.flavus* and *A.ochraceous* on the cap length of *P.tuber-regium* while 50ppm $CuSO_4$ against *A.flavus* and *A.ochraceous*; 100ppm $CuSO_4$ against *A.niger*, *A.flavus* and *A.ochraceous* and 150ppm $CuSO_4$ against *A.niger* was not significantly different from one another. For the stipe length, there was no significant difference for 150ppm $CuSO_4$ against *A.flavus* and *A.ochraceous*; 100ppm $CuSO_4$ against *A.niger* and *A.ochraceous* as well as 50ppm $CuSO_4$ against *A.flavus* and *A.ochraceous*. the untreated (control) for the chemical concentration do not produce significant difference on the pathogen (*A.niger*, *A.flavus* and *A.ochraceous*) thriving on the cap and stipe length of *P.tuber-regium* (Table 4).

Plates: Photograph of *Pleurotus tuber-regium* fruitbodies inoculated with *Azadirachita indica,Trichoderma harzanium* and Copper sulphate.

Discussion and conclusion

A. niger, A. flavus and *A. ochraceous* were the most frequently pathogens isolated from mushroom fruitbodies. The morphology and culture characteristic of fungal isolates observed from infected mushroom samples were in accordance with the reports of Obire and Amadi (2013).

Moulds make the fruitbodies unhealthly and cause damage associated with mushroom cultivation due to rot. The rot symptoms are visible as the fruit bodies grow. Jonathan *et al*., 2014 reported that mushroom green moulds were caused by *Aspergillus* spp., *Penicillium* sp., *Rhizopus* sp., *Ssicaria* sp. The soft rot of stipe and cap of the fruitbodies of *P. tuber-regium* observed, supported the findings of Ihejirika *et al*.,2005 that *Aspergillus* spp. caused soft rot of yam tuber and corns of *Colocasia esculenta.*

The in vivo evaluation showed that the concentration at 320gl for the plant extract was significantly ($P<0.05$) higher than other concentration levels (240g/l and 160g/l) in the control of rot disease of the stipe ad the cap *Pleurotus tuber-regium*. *A. indica* (Neem) leaves demonstrated strong effectiveness against the development of many diseases causing fungi through the soil or by its direct application (Manika *et al*., 2013). This antifungal activity of neem extract could be due to the presence of active chemicals such as Azadirachtin, Nimbidin, Nimbinin, Nimbolidin, Nimbin (Amadioha, 2002; Brahmachari, 2004). Similar results had also been reported that *N. tabacum, A. barbardensis* and *A. indica* extracts possessed fungicidal properties at different concentration levels on yam tuber dry rot diseases (Eze *et al.,* 2014).

*Trichoderma s*pecies are good sources of various enzymes such as exo- and endo-glucanases, cellulases, chitimase and growth stimulators which are responsible for its effectiveness as biocontrol agent (Mari Aidemark *et al.,* 2010).

It can be inferred from this result that 2×10^{10} spores/ml could be an effective concentration for *Trichoderma* spp. in-vivo applications. Although, it was significantly different from

the other concentration levels (1×10^6 and 1.5×10^8spores/ml). This agreed with the observation reported by Peluola (2005) that *Trichoderma* are bioagents for the protection of various seed and soil borne diseases of crops. In screen house experiment, $CuSO_4$ at 250ppm was active against *Aspergilus* spp. compared to other concentration levels on the length of cap and stipe of the mushroom. It was noted that the effect of $CuSO_4$ at 250ppm against *A. ochraceous* was significantly higher than *A.niger and A.flavus*. Fasidi *et al*., 2008 also observed that fungi differ significantly in morphology and physiology from one another, and they may be combated by compound of low toxicity for other organisms.

This study has established the fact that the fungal species isolated frequently from the stipe rot and cap rot diseases of fruitbodies of *P. tuber-regium* were *A. niger, A. flavus* and *A. ochraeous*. Mushroom farmers are used to the application of fungicides in controlling diseases of mushrooms, but it is noteworthy that both botanicals and microbial agents have great potentials at controlling the rot fungal diseases because they are natural occurring substance, environmentally friendly and affordable at less cost to the farmers.

RECOMMENDATION AND SUGGESTION

The ease of antagonistic ability, cultivation rapid growth and production of inhibitory effect on the mycelial of the pathogens suggest that *Trichoderma* spp. are promising isolates for use as biocontrol agent. *A.indica* can be used to control cap rot and stipe rot diseases of mushroom. Also, copper sulphate could be regarded as a broad spectrum pesticide in cultivation of *P. tuber-regium*. Hence they are recommended to mushroom farmers for use. The mixed formulation of the bioagent, botanical extracts and chemical could be integrated in management of mushroom diseases.

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