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In vitro and *In vivo* Control of Fungal Cobweb Disease of *Pleurotus ostreatus* (Jacq), Using Organic Materials

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Assessment of *In vitro* and *In vivo* Control of Fungal Cobweb Disease of *Pleurotus ostreatus* (Jacq), using organic materials.
Study Design: This study was designed to control the disease by using plant materials as organic control agent; both *In vitro* and *in vivo*.
Methodology: In *Pleurotus ostreatus*. In the *In vitro* test; food poison method was used while the *in vivo* test was undertaken by inoculation of fully colonized substrate bags of *Pleurotus ostreatus* infected with cobweb disease with crude extracts of *Piper guineense* and lime juice as treatment stock.
Results: The results indicated *P. guineense* inhibition of the growth of the pathogen at 22mm while lime juice did not show any impact on the growth of the pathogen. Additionally, *P. guineense* inhibited the growth of the pathogen in fully colonized but infected substrates bags of *P. ostreatus* invivo; such that the treated samples grew and developed the fruiting bodies of the mushroom, 3 days after treatment; whereas the one treated with lime juice developed the cobweb disease.
Conclusion: The study showed that effectiveness of *P. guineense* extract on the pathogen was possibly due to the bioactive chemicals such as phenols, tannins, flavonoid etc which concentration in the plant material occurred in surplus. These may have interfered with the molecular targets of the pathogen and caused it to loose cellular integrity and leakage of cell content. Alternatively, the study revealed that lime juice was ineffective; possibly because of the solvent used for it's

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preparation which could not enhance the release of the essential bioactive chemicals in lime juice for anti fungals. Consequently, this study recommends the use of *P. guineense* to mushroom farmers against fungal cobweb disease of oyster mushrooms.

Keywords: In vitro; in vivo; cobweb disease; Pleurotus ostreatus; bioactive chemicals; piper guineense; lime juice.

1. INTRODUCTION

Mushrooms are macrofungi with distinctive fruiting bodies of higher fungi such as Basidiomycota and Ascomycota; epigeous or hypogeous. They are large enough to be seen with naked eyes and picked by hand. Mushrooms have been used as food by mankind, since the stone age [1-3]. Mushrooms are a rich source of nutrients, particularly proteins, minerals, vitamins as well as bioactive constituents such as phenolic compounds, terpenes, steroids and polysaccharides [4]. They are known for good quality amino acids, vitamin B complex, sodium, potassium, iron and dietary fibres. They are considered as the primary natural sources of ergosterol or provitamins [5,6]. Mushrooms are also accredited with medicinal benefits imbedded with pharmacological effects such as antiviral, antioxidant, antitumor hypo-cholesterolemic and hypoglycemic [7]. Edible mushrooms are also reported to be found effective in reducing stress, cholesterol, asthma, diabetes, cancer and insomnia etc [8,9].

A Fungal cobweb disease of cultivated mushroom is commonly reported to cause dramatic mushroom crop failure and chemical control of the disease is undesirable due to residual effects.

Mushroom survival and multiplications lie on a number of factors which may act singly or have interactive effects among themselves [10,11].

Intensive cultivations of edible mushrooms can often be affected by some microbes such as some fungi, bacteria etc that rather frequently cause dramatic production loss. These infections are facilitated by the particular conditions under which the mushroom cultivation is carried out; such as warm temperatures, high humidity, carbon dioxide levels. Mushrooms, like all other crops are also affected adversely by a large number of biotic and abiotic agents/factors. Among the biotic agents are fungi, bacteria, viruses, nematodes, insects and mites that cause damage to mushrooms directly or indirectly [12].

A number of harmful fungi are encountered in compost and casing soil during cultivation of mushrooms. Many of these acts as cobweb disease, competitor molds, thereby adversely affecting spawn run; whereas others attack the fruit bodies at various stages of the crop growth producing distinct disease symptoms. Most times, there is complete crop failure depending upon the stage of infection, quality of compost and environmental conditions [4,13,14].

Cobweb disease of mushrooms is caused by several species of a fungal genus, *Cladobotryum Nees emend* (Syn. *Dactylium Nees*). They correspond to the conidial or asexual stage of species from the *Hypomyces* (Fries) L. R. Tulaone (Ascomycota, Hypocreales, Hypocreacea).

It is reported to appear at the end of a crop cycle; first as small white circular patches that appear on casing soil or basiciomas. They quickly spread by the grey-white mycelium that resembles a spider web [15]. As the mycelium sporulate producing masses of spores that are easily released when physically disturbed mainly by watering or picking operations; air currents from air-conditioning systems also sufficiently cause strong mobilization on the harmful spores [16]. Once released, the conidia spread through mushroom facilities by air currents to form secondary colonies on casing layers [5].

Cobweb disease is reported to be caused by several species of fungi in the genus *Cladobotryum. which* is known as one of the most infectious pathogen of mushrooms.

Its infections leads to the formation of patches of white cobweb-like mycelium [17,18]. As the disease develops, the first symptom is the appearance of white patches on the basidioms and then spreads quickly by means of fine gray white mycelium that resembles spider web. Fruiting bodies that are severely infected show discolouration and rottening. As the disease advances, dry spores begin to be released from the mycelium and spread to other basidiomes

with the help of various agents such as air current, sprinklers etc [16].

Prevention of cobweb disease is very paramount to preclude the emergence of the disease and reduce its impact peradventure it has already occurred. Regular cleaning of the mushroom house; lowering the RH in the cropping house and increase in air circulation in the cropping house will go very far to preclude the occurrence of cobweb disease. These are possible based on the report of Sharma [19].

In order to control or manage cobweb disease, a method employed should prevent dispersion of conidia as reported above; hence this is the main way leading to the infection [20-23].

The end of the crop cycle is very important for the removal of any residual disease. The wet conidia of *Cladobotryum* species could be destroyed at 45^0C for at least 3 mins; but they resist higher temperatures of about 100^0C when dry. However, the pathogenic mycelium is susceptible to a 15 mins, (ie 40^0C for 15 mins) when dry [21]. Although thermal disinfection at the end of a crop cycle by cooking out (about 65 – 70^0C for 9 – 12 hours) still stands as one of the most important strategy to ensure the mushroom crop is very disinfected.

There are reports that some farmers still employ chemical control strategy over cobweb disease. No matter how effective a chemical may be, its application is undesirable because of its residual impact and hazardous effect on consumers.

Due to consumer and the environmental concerns; there is a very strong pressure to reduce or preclude the use of chemical pesticides. This has led to the intensification of organic control in Agriculture, for safe alternatives by the use of plant materials.

This then calls for safe alternatives possibly with plant materials.

Interestingly, it is reported that many plants with bioactive potentials act as organic or biofungicides. Compost tea from spent mushroom substrate and essential oils from plants have been tested as alternatives. Many aromatic plants with bioactive chemicals are better proven as safe alternatives. Plant materials such as (Lime juice), *Piper guineense, Xylopia aethiopica,* Trametes and Sclerotia powder have been reported to contain some bioactive constituents [24-27].

However, it is reported by Inga and Alexander [28] that the tested natural preventatives are *Oreganum vulgare,* essential oils, carvacrol, thymol eugenol and trans-cinnamaldehyde. These are the most tested organics with good antifungal effects against some storage fungi. In the other hand, Ibukun et al. [29] also submitted that the potency of lime juice is being enhanced by the type of solvent used, which indicates that there are some active ingredients in the lime juice that have antimicrobial/antifungal effect which will not be released except when lime fruit is used in conjunction with a particular solvent.

Ibunkun et al. [29] reported that the potency of lime juice is dependent on the type of solvent used to extract it. And that there are some solvent that can enhance the release of bioactive chemicals for antifugals in lime juice.

These plants have been reported to contain bioactive compounds such as tannins, flavonoids, essential oils and phenolic acids [30]. Davidson [31] reported that tannins affect fungal pathogens directly on the cell membrane by metal depletion.

Harris and Dennis [32,33] reported that terpenoid type of bioactive chemical caused the zoospores of *Phytophthora* spp to develop cytoplasmic granulations and disruptions of the pathogen's plasma membrane and leakage of cellular contents.

According to the report of Cowan [30], tannins can bind to the pathogen's protein enzymes to inhibit the enzyme and cause substrate deprivations; while alkaloids can interact into the cell wall for disruption and cause leakage of cellular contents. He also suggested that flavonoids and phenolic acid bind to adhesion and complex with the fungal cell wall and inactivates fungal enzymes.

Consequently, the aim of this study is to isolate, and identify the fungal species responsible for cobweb disease in *P. ostreatus;*as well as proffer strategy for the management of the disease in vivo and *In vitro* using plant materials.

2. MATERIALS AND METHODS

The study was carried out in the laboratory of the Department of Plant Science and Biotechnology of Rivers State University. And the sample was

Plate 1. Fully Colonized mushroom substrate infected with cobweb disease

procured from Dilomat Mushroom farm located in the campus. The samples were fully colonized or ramified mushroom substrates of *Pleurotus ostreatus,* infected with fungal cobweb disease obtained from the Dilomat mushroom farms and taken to the laboratory for study.

2.1 Sample Preparation

A 10g quantity of the infected substrates was obtained mechanically using a sterile inoculating loop into a test tube; from which the infectious stock was prepared for inoculation.

2.2 Preparation of Normal Saline for Serial Dilution

A quantity of 8.5g of analytical salt (NaCl) was dissolved in 1 litre of distilled water. The diluent was sterilized in an autoclave at 121^0C Psi for 15 min [34].

2.3 Serial Dilution/Inoculation

Exactly 9ml of normal saline was dispensed into different test tubes, then 10 fold serial dilution was made, in which 1ml from the stock solution was transferred from 10^{-1} to 10^{-3} and also transferred direct.

2.4 Media Preparation (Sabouraud Dextrose Agar – SDA)

This was prepared according to the manufacturer's prescription by weighing 65gm of SDA powder and dissolving in 1 litre distilled water. Mass volume relationship was used to compute the actual required measurements. The mixture was shaken vigorously and sterilized by autoclaving at 121^0C Psi for 15 min. Antibiotic was added to prevent bacterial contaminations on cooling, it was dispensed into sterile Petri dishes [35].

3. ORGANIC MATERIALS AS CONTROL AGENT

The plant materials used were lime juice and dry seeds of *Piper guineense.* The crude extracts of the pulp of lime and the dried seeds of *P. guiueense* were made and used as food poison into the agar wells of the SDA.

3.1 *In vitro* Test by Agar well Diffusion Method

The crude extract of plant material, lime juice was used as a control agent of the disease: Inoculum was collected with inoculation needle and added to already prepared broth: The agar

plate surface was then inoculated by spreading a volume of the microbial inoculum over the entire agar surface and a cork borer was used to create wells on the agar plate and the plant extracts were poured into the agar wells and incubated for 48 hours. This was observed to determine the possible inhibitions by the organic extract.

3.2 *In vivo* Test

Two fully ramified or colonized *P. ostreatus* mushroom substrate bags which were infected with cobweb disease were inoculated with the plant extracts separately per treatment (lime juice and the extract of *Piper guineense*) in the PSB laboratory. A sterilized knife by flaming red-hot and cooking was used to create holes on the mushroom substrates. 5ml each of the extracts were placed per treatment into each of the holes and incubated. The treated substrate bags were kept away from the rest uninfected substrates. These were observed daily for possible results.

3.3 Phytochemical Screening of the Plant Materials

The phytochemical screening of the plant materials used as control agent for various bioactive chemicals were conducted using standard procedures as prescribed by Soforora [36]; Trease and Evans [37].

3.4 Test for Alkaloids

Extracts of the plant materials were dissolved separately, each in dilute Hydrochloric acid (HCL) and filtered. The filtrates were treated with Mayer's reagent (Potassium mercuric iodide. Formation of a yellow coloured precipitate was an indication of the presence of alkaloids.

3.5 Test for Terpenoids

Salkowski method was used to determine the presence of terpenoids. The crude extracts were separately shaken with 2ml chloroform; followed by addition of concentrated 2ml of H_2SO_4 along the sides of the test tube. A reddish – brown colour of the interface indicated the presence of terpenoids.

Two methods were used to test for tannins;

a) To a 10ml freshly prepared 10% KOH in a beaker; 0.5ml of each of the extracts were added and shaken to dissolve. A dirty precipitate observed indicated the presence of tannins.
b) 0.5ml of the extracts were boiled in 10ml water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and the solution observed for brownish green or blue black colouration.

Plate 2. Infected and colonized mushroom substrates, also treated with plant extracts

3.6 Test for Saponins

About 0.5ml of the extracts were added to 5ml distilled water in a test tube and the solutions shaken vigorously and then were observed for a stable persistent froth. The frothings were mixed each with 3 drops of olive oil and shaken vigorously, after which the experiments were observed for the formation of an emulsion.

3.7 Test for Steroids

Exactly 1ml of the extract was dissolved in 10ml of chloroform and equal volume of concentrated H_2SO_4 was added by the sides of the test tubes. The upper layer turns red and H_2SO_4 layer showed yellow with green fluorescence, an indication of the presence of steroids.

3.8 Test for Flavonoids

Two methods were used to test for flavonoids.

a) A portion of the extracts was heated with 10ml of ethyl acetate over a steam bath for 3 minutes; the mixtures were filtered and 4ml of the filtrates were shaken with 1ml of ethyl acetate over a steam bath for 3 minutes; the mixtures were filtered and 4ml of the filtrates were shaken with 1ml of dilute ammonia solution. A yellow colouration confirms the presence of flavonoids.
b) Dilute 5ml ammonia was added to a portion of an aqueous filtrate of the extracts. Then 1ml Concentrated H_2SO_4 was added. A yellow colouration indicated the presence of flavonoids.

The extracts were stirred each with 10ml distilled water and then filtered. A few drops of 5% ferric chloride was added. Black or blue-green colouration or precipitate was the confirmatory test for phenols.

4. RESULTS AND DISCUSSION

The results of this study are presented on Plates 3 – 5 and Table 1.

The results of the invitro test showed that the treatment of the sample with the crude extract of *Piper guineense* in the agar wells inhibited the growth of the cobweb propagules with inhibition growth measurement of 22mm; while the lime juice did not show any impact on the disease propagule *In vitro*.

The in vivo results also showed that lime juice could not inhibit the growth of the cobweb disease after treatment. However, treatment with *P. guineense* inhibited the growth of the cobweb disease in vivo and allowed the growth and development of the mushroom fruiting bodies; 3 days after treatment. This is shown in Plate 3.

The results of the phytochemical screening test are presented on Table 1. The results revealed that alkaloid and flavonoids were absent in both *P. guineense* and lime juice used as control agents against the fungal cobweb disease. It also revealed that tannins and saponins are present in the seed extract of *P. guineense*, but absent in lime juice, although it contained terpenoids which was shown absent in *Piper.* However, the phytochemical results also revealed surplus of steroids and phenol in both plant extracts.

The results of this study indicated that the plant extract of *Piper guineense,* used as food poison in agar wells had an impact of inhibition on the fungal cobweb pathogen, with a growth inhibition of 22mm. Alternatively, the lime juice used in same pattern as in above did not have any inhibitory or destructive impact on the spread of the pathogen.

However, the in vivo result showed that *P. guineense* seed extract administered on fully colonized substrate bags of *Pleurotus ostreatus* inhibited the growth and development of the cobweb disease and gave room for the growth of the mushroom fruiting bodies; 3 days after treatment. The result of the phytochemical screening of the plant extracts indicated that alkaloids and flavonoids were absent in the plant extracts; while tannins and saponins were absent in the lime juice; although steroids and phenols occurred in surplus quantities in the two plant extracts.

It is possible that *P. guineense* was effective because of the presence of the bioactive chemicals contained therein. The bioactive chemicals may have acted by inhibiting cutinases and lacases of the pathogen; this to conform to the report of Nmom and Ajuru [22]; who reported that saponins and tannins inhibit cutinases and lacases of pathogenic fungi. It is also likely that the bioactive compounds may have acted directly on the fungal cell membrane by metal depletion,

in line with the suggestions of Davidson [31]; that tannins affect fungal pathogens directly on the cell membrane by metal depletion. It is also possible that the extract of *P. guineense* interfered with the molecular targets of the pathogen's tissues. Since steroids and phenols occurred in surplus quantity in the *P. guineense* extract. It could be that they interfered with the fungal membrane integrity and possibly complexed with the sugar residues of the bioactive chemicals. This seems to clearly agree with the report of Keykens et al. [38] that; steroidal glycol alkaloids interfere with fungal membrane integrity and complexes with the sugar residues of saponin molecules with the pathogen.

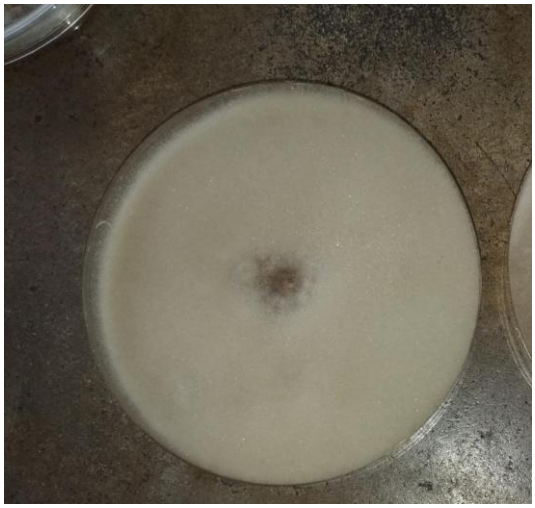

Plate 3. Petri dishes bearing agar wells and showing inhibitory growth of cobweb propagule by *Piper guineense*

Plate 4. Showing no impact on the disease propagule by lime juice treatment and no inhibitory effect

Table 1. Showing Phytochemical Screening of *P. guineense* and lime juice for the treatment of cobweb disease in *Pleurotus ostreatus*

S/No	Phytochemicals	*Piper guineense*	Lime juice
1.	Alkaloid	_	_
2.	Terpenoids	_	+
3.	Tannins	+	_
4.	Saponins	+	_
5.	Steroids	++	++
6.	Flavonoids	_	_
7.	Phenol	+++	+++

Plate 5. Successful treatment of cobweb disease with *P. guineense* and showing growth of mushroom fruit bodies, 3 days after treatment

Most importantly, the bioactive compounds may have also acted by causing the fungal spores to develop cytoplasmic granulations and disruptions of the plasma membrane; thereby leading to leakage of cellular contents; in accordance with the submissions of Harris and Dennis [32,33]. The presence of tannins may have caused its binding to the pathogen's protein enzyme and as a result, inhibited its enzymatic actions. This is also in line with the report of Cowan]30]. Also in line with his report, is that, it is likely that the chemicals of the flavonoids and phenolic acid may have bound to adhesion and complexed with the fungal cell wall and inactivated the pathogen's enzymes.

For the ineffectiveness of the lime juice *In vitro* and in vivo; it is obvious as was reported earlier in this study, that lime juice is not one of the reportedly tested natural preservatives, as was reported by Inga and Alexander [28]. Additionally, it is possible that the solvent used in this study for juicing is not such that could release, the bioactive chemicals in the lime. This therefore implies that the potency of the lime juice in this study was not enhanced due to the type of solvent used to juice the lime. This implies that, if the right solvent was used, there would have been an enhancement of the release of accompanying bioactive chemicals for antifungals; as was suggested by Ibukun et al. [29].

5. CONCLUSION

The results of this study establish that *Piper guineense* extract inhibited the growth of the fungal cobweb pathogen; Cladobotryum mycophilium invitro and also inhibited the growth of the pathogen in fully colonized substrate bags of *Pleurotus ostreatus;* such that the treated samples grew and developed the mushroom fruiting bodies; 3 days after treatment whereas lime juice did not show any impact on the growth of the pathogen, invitro and in vivo. This shows that treatment of cobweb disease with the seed extract of *P. guineense* is effective.

This study also has established that the efficacy of the extract of *P. guineense* was possible due to the bioactive chemicals; such as Phenols, tannins, flavonoids which concentrations in the plant material occurred in surplus. These may have interfered with the molecular targets of the pathogen and caused it to loose integrity thus causing cell leakage. Alternatively, the study also has established that lime juicing which could not enhance the release of essential bioactive chemicals for antifungals could do better if an appropriate solvent is used.

Conclusively, fungal cobweb disease of oyster mushroom can be managed, using organic material as a good alternative to field inventory to enhance agricultural sustainability of oyster mushroom cropping.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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