

Original Research Article

Possible Antibiosis Effect of the Metabolites of Three Fungal Species Resident in Rice Straw and Husk Compost on the *in vitro* Radial and Vegetative Growth by *Pleurotus ostreatus* Strain EM-1 and *P. eous* Strain P-31

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ABSTRACT

The inhibition of one microorganism by another through chemical means (antibiosis) or by competition for nutrient in a micro-ecological environment is a well-known phenomenon in mushroom composts during preparation of substrate for bioconversion into fruiting bodies. The effect of culture metabolites of three resident fungi (*Aspergillus flavus*, *Penicillium citrinum* and *Trichoderma harzianum*) in rice straw and husk on growth of mycelium of *Pleurotus ostreatus* and *P. eous* was studied *in vitro* using the radial growth and dry weight accumulation method in solid and liquid media respectively. Estimation of radial growth and dry weight of the mycelium was carried out in Potato Dextrose medium amended with 1:1 - 1:10v/v dilutions of the cultural filtrates. The antibiosis test showed that the cultural filtrates of the three respective test fungi variably depressed radial and vegetative growth of *P. ostreatus* and *P. eous* on agar and liquid medium respectively. The antibiosis effect was severer on *P. ostreatus* than *P. eous*. *T. harzianum* culture metabolite was the most potent completely preventing radial growth of both oyster mushrooms at all concentrations tested (1:1 - 1:10v/v). The antibiosis effect of the metabolites of the test fungi on growth of *P. ostreatus* and *P. eous* can be ranked as follows (in decreasing order) *T. harzianum* > *A. flavus* > *P. citrinum*. The estimation of dry matter accumulation by the oyster mushrooms in the presence of the culture metabolites gave the same trend except that increasing dilution of the cultural filtrates permitted feeble growth of the mycelium of both *Pleurotus* species but never approximated the dry weight obtained in the control. The highest concentration of culture filtrates of the three test fungi (1:1v/v dilution) depressed vegetative growth by 5-6 times. Thus the test fungi *A. flavus*, *P. citrinum* and particularly *T. harzianum* may adversely affect economic productivity of the mushrooms if found in high population in the compost. The practical implications of the findings are discussed.

Keywords

Antibiosis,
Culture
metabolites,
*Aspergillus
flavus*,
*Penicillium
citrinum*,
*Trichoderma
harzianum*,
Rice straw
and husk,
Vegetative
growth,
*Pleurotus
eous*,
*Pleurotus
ostreatus*

Introduction

Compost substrate for mushroom cultivation must have certain physical qualities and must support aerobic conditions, hold water without becoming waterlogged and have a proper pH and good drainage (Buswell, 1984; Piet *et al.*, 1990; Oei, 1991; Obodai *et al.*, 2010). The aim of commercial mushroom substrate preparation is to produce a substrate that is optimal and selective for vegetative mycelial growth. Biologically, the substrate must have a population of suitable microorganisms and during the growth of these microorganisms in the compost there is the production of secondary metabolites by these microorganisms. Imperfect fungi are among microorganisms that grow in the compost and may compete for nutrients and space and therefore there will be a form of antagonism between these fungi. This antagonism may be in the form of competition for nutrients, chemical antibiosis and lysis of mycelium (Thomas and Alma, 1984; Morris *et al.*, 1995; Lopez-Arevalo *et al.*, 1996; Seaby, 1996; Jayalal and Adikaram, 2007; Obodai and Odamtten, unpublished data). Antibiosis is the inhibition of one microorganism by the metabolic product of another. Although it is usually an inhibition of growth and sporulation, it may be lethal. The metabolite penetrates the cell and inhibits by chemical toxicity. Lysis is destruction and decomposition of biological materials by enzymes of the parasite (Mumpuni *et al.*, 1998; Goltapeh *et al.*, 2000; Obodai *et al.*, 2010; Odamtten, Obodai and Odamtten, unpublished data). Contaminants are described as organisms that are undesired in mushroom cultivation since these species affect the growth and development as well as the quality of the mushroom crop. Hence many researchers (Sharma and Kumar, 2008; Reyes *et al.*, 2009; Singh *et al.*, 2010;

Chakraborty *et al.*, 2013) described contaminants as competitor weeds. Contaminants are primarily moulds, bacteria, viruses and insects and can be divided into two well defined groups; those attacking the mushrooms are called pathogens while those competing for the substrate are known as indicators or competitors (Cailleux and Diop, 1978; Sandhu and Sidhu, 1980; Rangunathan *et al.*, 1996; Rajarathnam *et al.*, 1997 and Jandaik *et al.*, 1998).

In general, mushroom pathogens are not as numerous as the competitor moulds, though they can be much more devastating as all moulds and bacteria are damaging to the mushroom crop. On the contrary, several are beneficial. These cannot be called true "contaminants" since cultivators try to promote, not hinder their growth. Examples of yield enhancing organisms are several thermophilic fungi and bacteria, including *Humicola*, *Torula*, *Actinomyces* and *Streptomyces* selected *Pseudomonas* and *Bacillus* species. The objective of this study was to investigate the *in vitro* effect of culture metabolites of three contaminant resident fungi in rice straw and husk compost (*Aspergillus flavus*, *Penicillium citrinum* and *Trichoderma harzianum*) on the radial and vegetative growth of two *Pleurotus* species (*P. eous* Strain P-31 and *P. ostreatus* Strain EM-1). This information could be helpful in elucidating their role in the growth and bioconversion of the oyster mushroom in compost substrate such as rice straw and husk.

Materials and Methods

Mushroom cultures and maintenance

Cultures of *Pleurotuseous* (Berk.) Sacc. (Strain P-31) and *P. ostreatus* (Jacq.Ex.Fr) Kummer (Strain EM-1) were obtained from

the National Mushroom Mycelium Bank at the Food Research Institute, under the Council for Scientific and Industrial Research, (CSIR-FRI), Ghana. Stock cultures of *P. ostreatus* and *P. eous* were grown on slants of either Potato Dextrose Agar (PDA) in McCartney tubes or in Petri dishes and were kept in a refrigerator at $8\pm 1^{\circ}\text{C}$. These individual cultures were subsequently sub-cultured every two weeks before use. All media used were sterilized at $1.05\text{kg}/\text{cm}^3$ pressure for 15 minutes.

Preparation of potential antagonistic metabolites

The potential antagonists: *Trichoderma harzianum*, *Penicillium citrinum* and *Aspergillus flavus* were chosen because of their preponderant occurrence during the composting and cultivation process of two *Pleurotus* species. To obtain the culture metabolites of the different potential antagonists, 3cm agar disc of *Aspergillus flavus*, *Penicillium citrinum* or *Trichoderma harzianum* were inoculated into a 500ml Erlenmeyer flask containing 250ml of Potato Dextrose Broth (PDB). There were four replicates of each test fungus. Incubation was at $30\pm 2^{\circ}\text{C}$ for 7 days. The filtrate was collected into sterile flasks using the conventional dry weight method of collecting mycelium on a previously weighed filter paper at the end of the incubation period. The filter paper with the mycelium was dried at 75°C for 24 hrs and reweighed. The collected filtrate was then filtered through a sterile Acrodisc[®] Millipore filter $0.2\mu\text{m}$ (Gelman Sciences, USA). The undiluted filtrate served as the stock from which different dilutions of 1:1, 1:2; 1:5 and 1:10 v/v were prepared to amend either Potato Dextrose Agar (PDA) or Potato Dextrose Broth (PDB) media. The pH of the medium was ascertained using a pH meter (pHM92 Lab pH meter

(MeterLab[™], Radiometer Analytical A/S, Copenhagen, Denmark).

In vitro assessment of test fungal culture filtrates on their antibiosis effect on the vegetative growth of the two *Pleurotus* species (*P. eous* and *P. ostreatus*)

The antibiosis effect was assessed by two methods, radial growth on agar and the dry weight method. In order to study antibiosis effect of the three fungi (*A. flavus*, *P. citrinum* and *T. harzianum*) on radial growth of the mushroom a modification of the 'Food poisoning technique' (Dennis and Webster, 1971 and Mondal *et al.*, 1995) was followed. In this method a known volume of either the PDA medium was poured into each of the sterilized Petri dishes. Medium in Petri dishes was allowed to cool and just before solidification, a known volume of each of the crude filter-sterilized metabolites was added separately and then mixed homogeneously to obtain 1:1, 1:2; 1:5 and 1:10 v/v of the respective culture filtrates. The basal PDA amended with varying dilutions of the culture filtrates were inoculated at the centre of the plate with a 3mm active mycelia agar disc of either *P. eous* or *P. ostreatus* in triplicates for each dilution level of treatment. Radial vegetative growth of the mycelium of the mushrooms on the solid culture medium was assessed by measuring the growth of the fungus along two diameters drawn at right angles at the bottom of the Petri plates prior to inoculation. Measurements were made every three days until complete colonization of the Petri plates was obtained in the control after 12 days.

Vegetative growth in liquid culture medium was assessed by determining the dry weight of the harvested mycelium at the end of 7 days incubation period in varying dilutions (1:1 – 1:10v/v) of the culture filtrate of the

test fungi. Thirty (30) millilitres of the prepared basal medium was poured into 250ml Erlenmeyer flasks and was inoculated with a 3mm active mycelia agar disc in triplicate for each dilution level of treatment. The unamended Potato Dextrose Broth served as control. Both Petri plates and Erlenmeyer flasks were incubated at $30\pm 2^{\circ}\text{C}$ for 7 and 12 days for radial and vegetative growth respectively.

Results and Discussion

Influence of the culture metabolites of the test fungi on radial growth of *P. eous* and *P. ostreatus*

Figs 1 and 2 show influence of the culture metabolites of the test fungi on radial growth of the oyster mushrooms. The metabolites from the three test fungi behaved differently. At the highest concentrations applied (1:1v/v) there was no growth of both *P. eous* and *P. ostreatus* mycelium in all the plates amended with the metabolites of the test fungi (Figs 1 & 2). The effect was severer with no growth on PDA amended with 1:2v/v dilutions of *A. flavus*, *P. citrinum* and *T. harzianum* inoculated with mycelium of *P. ostreatus* (Fig 2). Culture metabolites of *T. harzianum* was the most potent against radial growth of the two test *Pleurotus* species since it totally prevented radial growth of the mycelium at all dilutions tested (Figs 1 & 2). This was followed by the culture filtrate of *A. flavus* which was less potent with increasing dilution beyond 1:5v/v. The severity of the antibiosis effect can be ranked as: *T. harzianum* > *A. flavus* > *P. citrinum* decreasing order. Plates 1-3 show the radial growth of the two *Pleurotus* on PDA amended with the culture filtrate of *A. flavus* (Plate 1), *P. citrinum* (Plate 2) and *T. harzianum* (Plate 3). In Plates amended with the culture filtrates which permitted feeble

growth of both mushroom species, diameter of cultures never approximated that of the control and was less than one quarter (25%) of the control (*P. ostreatus*) and less than (45%) of the untreated control (*P. eous*) (Figs 1 & 2).

Vegetative growth in liquid medium

The antibiosis effect by the metabolites of the three fungi obtained on agar was replicated in liquid medium both following almost the same trend (Figs 3 & 4). Concentration of 1:1v/v depressed growth of *Pleurotus* by 5–6 times. The depressive effect of the metabolites on the two *Pleurotus* species declined with increasing dilution of the culture metabolites. However, vegetative growth in PDB amended with 1:10v/v dilution of the culture filtrate of *A. flavus*, *P. citrinum* and *T. harzianum* never approximated that of the control (Figs 3 & 4). Plates 4 and 5 show the trend in vegetative growth of *P. ostreatus* (Plate 4) and *P. eous* (Plate 5) in Potato Dextrose Broth amended with culture metabolite of *A. flavus* and *T. harzianum*. During the growth of *P. ostreatus* mycelium, pH drifted from 4.8–5.4 (*A. flavus*); 5.1–5.6 to 5.4–5.9 (*P. citrinum*) and pH 5.3–5.7 to 5.5–5.9 (*T. harzianum*) (Table 1). In the medium used for the cultivation of *P. eous* the pH drifted from 4.8–5.4 to 3.7–6.2 (*A. flavus*); 5.3–5.7 to 5.5–6.3 (*P. citrinum*) and pH 5.3–6.6 to 5.2–5.8 (*T. harzianum*) (Table 2).

Antibiosis is a well-known phenomenon in biological interaction where chemical antagonism between two or more organisms is detrimental to at least one of them or there is antagonistic association between an organism and the metabolic substances produced inhibits growth and sporulation of one which may be lethal. The compost for cultivation of mushrooms contains contaminants which are undesirable for

mushroom cultivation. During the process of composting the phenology of microorganisms including bacteria, actinomycetes, fungi and protozoa is different at different stages and different groups of microorganisms may dominate (Hayes, 1977).

The potential antagonistic fungi *A. flavus*, *P. citrinum* and *T. harzianum* were chosen for this present study because of their preponderance during the composting of rice straw and husk used for the cultivation of the two *Pleurotus* species. Some fungi which are harmful to *Pleurotus* cultivation have been identified in many commercial mushroom substrates including rice straw and rice husk. These include *Aspergillus fumigatus*, *A. terreus*, *Fusarium*, *Monilia*, *Penicillium*, *Sclerotium rolfsii*, *Coprinus cinereus*, *Mucor pusillus*, *Rhizopus microsporus*, *Chaetomium thermophile* and *Trichoderma* (Sandhu and Sidhu, 1980; Chang-Ho, 1982; Obodai, 1992; Lopez-Arevalo *et al.*, 1996). *Trichoderma harzianum* and *P. citrinum* are being recorded for the first time in Ghana as contaminants of mushroom compost. Obodai, (1992) showed that metabolites of *T. viride* were antagonistic to *P. ostreatus* and *P. sajor-caju*. *Penicillium cyclopium* also decreased fruit body emergence of *P. sajor-caju* by 50–75% (Jandaik *et al.* (1998). During the last decades, some members of the genus *Trichoderma* (*T. koningii*, *T. hamatatum*, *T. longibrachiatum*, *T. citreoviride*, *T. crassum*, *T. spirale* and *T. harzianum*) have been isolated from mushroom compost (Ospina-Geraldo *et al.* 1999, Jandaik and Guleria, 1999; Castles *et al.*, 1998). Aggressive colonization of mushroom compost causing epidemic outbreak of green mould was attributed originally to *T. harzianum* (Doyle, 1991; Morris *et al.*, 1995; Seaby, 1996, 1989, 1987). Recently, Jayalal and Adikaram, (2007) isolated *Trichoderma harzianum*

from mushroom compost causing green mould in oyster mushroom (*P. ostreatus*) resulting in considerable inhibition of growth of mycelium and formation of fruiting bodies thus lowering substantially the yield.

Results presented in this paper clearly show that the culture metabolites of *A. flavus*, *P. citrinum* and *T. harzianum* were antagonistic (to different extent) to the *in vitro* radial growth and dry matter accumulation by mycelium of both *P. ostreatus* and *P. eous*. The antibiosis effect of the metabolites of *T. harzianum* was the most potent and the antibiosis effect on the two *Pleurotus* species could be ranked as follows (in descending order): *T. harzianum* > *A. flavus* > *P. citrinum*.

Some workers Stamets (2000) and Narh *et al.* (2011) have shown that the best pH for growth of *Pleurotus* species is between pH 5.5–6.5. The pH range in the medium for testing antibiosis of the culture filtrates were within the optimum for the two *Pleurotus* species used in these studies. Therefore, the decline in the growth of the two *Pleurotus* species could be partly attributed to the active ingredients of the metabolites of the test fungi. For example, culture filtrate of *T. harzianum* at all concentrations tested (1:1 – 1:10v/v) completely prevented radial growth of both *P. ostreatus* and *P. eous* (Figs 1 & 2; Plates 1–3). This was followed by *A. flavus* and *P. citrinum* culture metabolites which was less potent with increasing dilution but growth in the lower concentrations of (1:5– 1:10v/v) never approximated that of the control. The estimation of the antibiosis effect of the metabolites on dry matter accumulation of *P. ostreatus* and *P. eous* gave similar trends. Vegetative growth of the two *Pleurotus* species in PDB amended with 1:1v/v was depressed by more than 5-6 times (Figs 4&5).

Table.1 pH of the culture medium amended with the indicated dilution of the culture filtrate of the test fungi used in the in vitro antibiosis test on *P. ostreatus* Strain EM-1

Dilution ratio (v/v)	pH of Culture Metabolite of test fungi					
	Initial	Final	Initial	Final	Initial	Final
	<i>Aspergillus flavus</i>		<i>Penicillium citrinum</i>		<i>Trichoderma harzianum</i>	
Control (Only PDB)	5.4±0.0	5.7±0.03	5.6±0.0	5.9±0.33	5.6±0.0	5.9±0.33
1:10	5.4±0.0	5.7±0.06	5.5±0.0	5.8±0.33	5.3±0.0	5.7±0.67
1:5	5.2±0.0	5.5±0.06	5.4±0.0	5.8±0.33	5.6±0.0	5.6±0.12
1:2	5.0±0.0	5.3±0.06	5.2±0.0	5.6±0.00	5.3±0.0	5.6±0.09
1:1	4.8±0.0	5.1±0.06	5.1±0.0	5.4±0.68	5.7±0.0	5.5±0.15

All values are means of five replicates S.E. – Standard Error (±) C.D. – Critical difference (p = 0.05)

Table.2 pH of the culture medium amended with the indicated dilution of the culture filtrate of the test fungi used in the in vitro antibiosis test on *P. eous* Strain P-31

Dilution ratio (v/v)	pH of Culture Metabolite of test fungi					
	Initial	Final	Initial	Final	Initial	Final
	<i>Aspergillus flavus</i>		<i>Penicillium citrinum</i>		<i>Trichoderma harzianum</i>	
Control (Only PDB)	5.4±0.0	5.5±0.0	5.6±0.0	5.5±0.0	5.6±0.0	5.8±0.09
1:10	5.4±0.0	4.8±0.06	5.3±0.0	6.0±0.0	5.4±0.0	5.5±0.03
1:5	5.2±0.0	5.6±0.0	5.6±0.0	6.1±0.0	5.3±0.0	5.3±0.03
1:2	5.0±0.0	6.2±0.0	5.3±0.0	6.3±0.03	6.3±0.0	5.2±0.07
1:1	4.8±0.0	3.7±0.0	5.7±0.0	5.4±0.07	6.6±0.0	5.2±0.03

All values are means of five replicates S.E. – Standard Error (±) C.D. – Critical difference (p = 0.05)

Fig.1 The effect of varying dilutions of cultural metabolites of indicated fungi on the radial growth of *P. ostreatus* on PDA at 30±2°C for 12 days

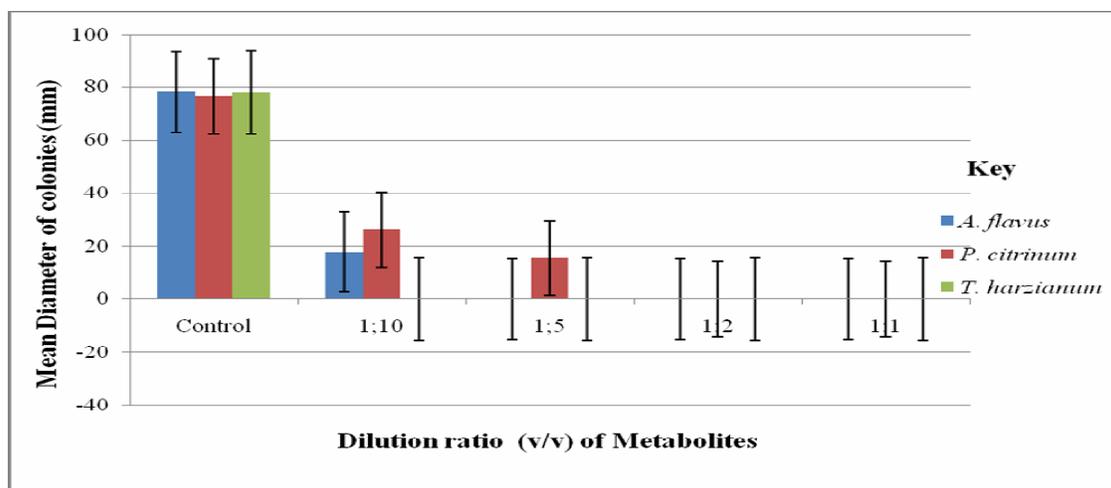


Fig.2 The effect of varying dilutions of cultural metabolites of indicated fungi on the radial growth of *P. eous* on PDA at 30±2°C for 12 days

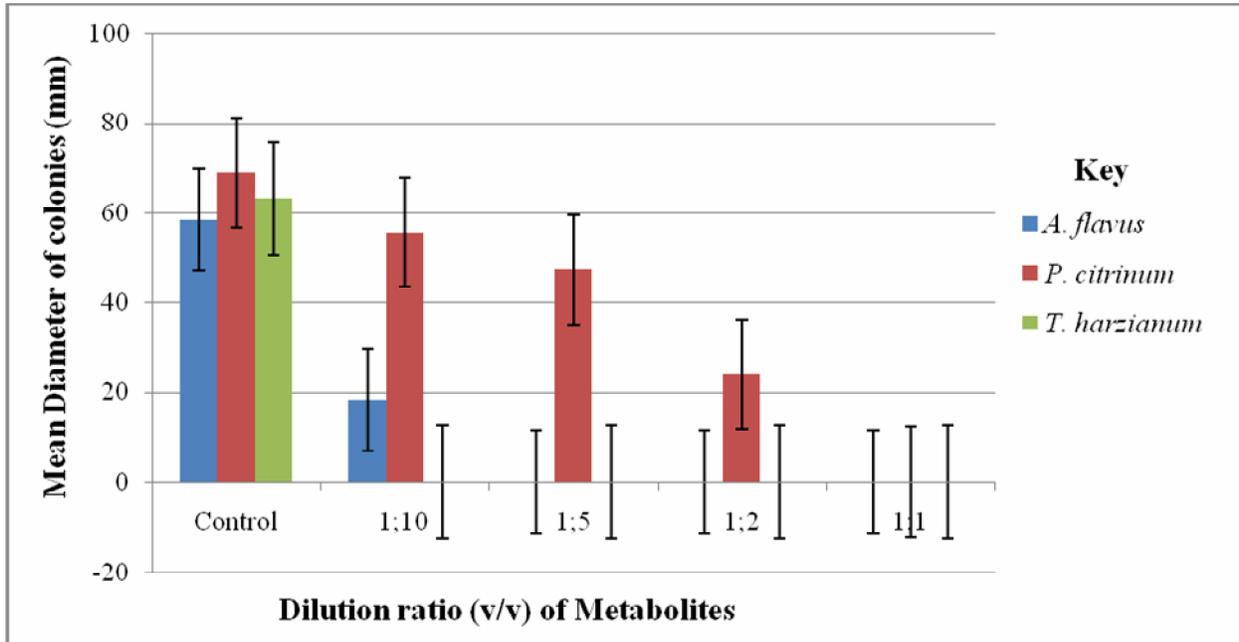


Fig.3 Influence of varying dilutions of the cultural metabolites of indicated fungi on the vegetative growth of *P. ostreatus* in PDB at 30±2°C for 7 days

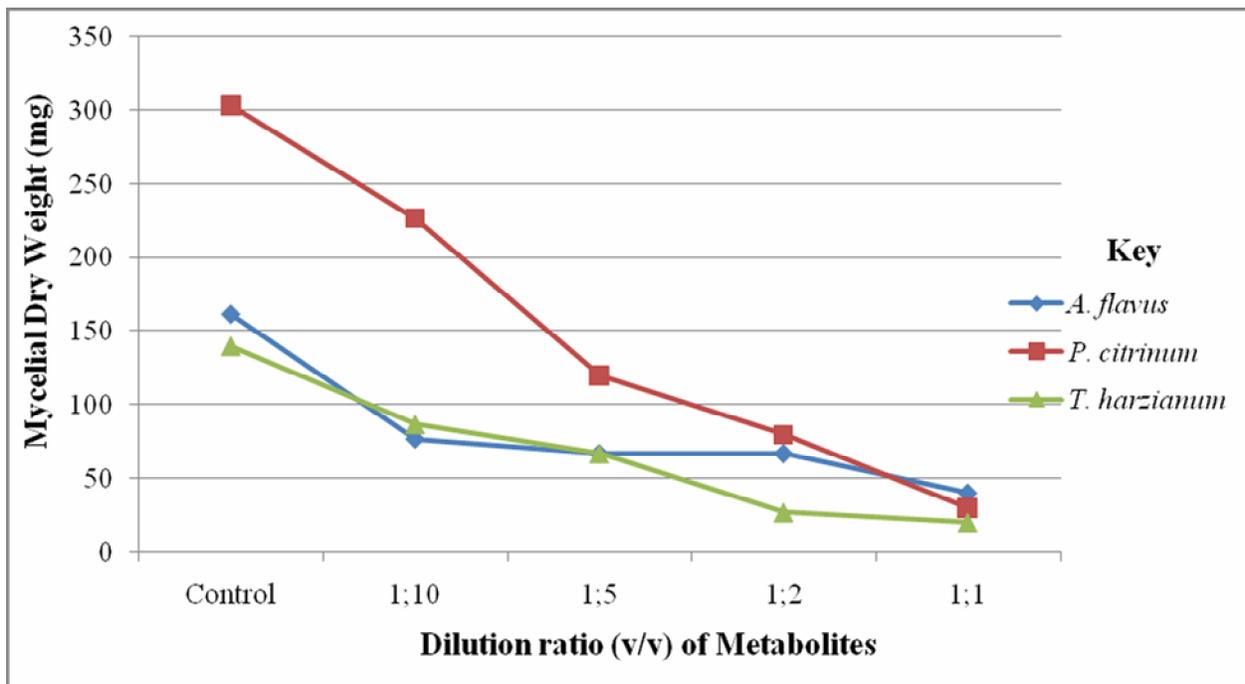


Fig.4 Influence of varying dilutions of the cultural metabolites of indicated fungi on the vegetative growth of *P. eous* in PDB at $30\pm 2^{\circ}\text{C}$ for 7 days

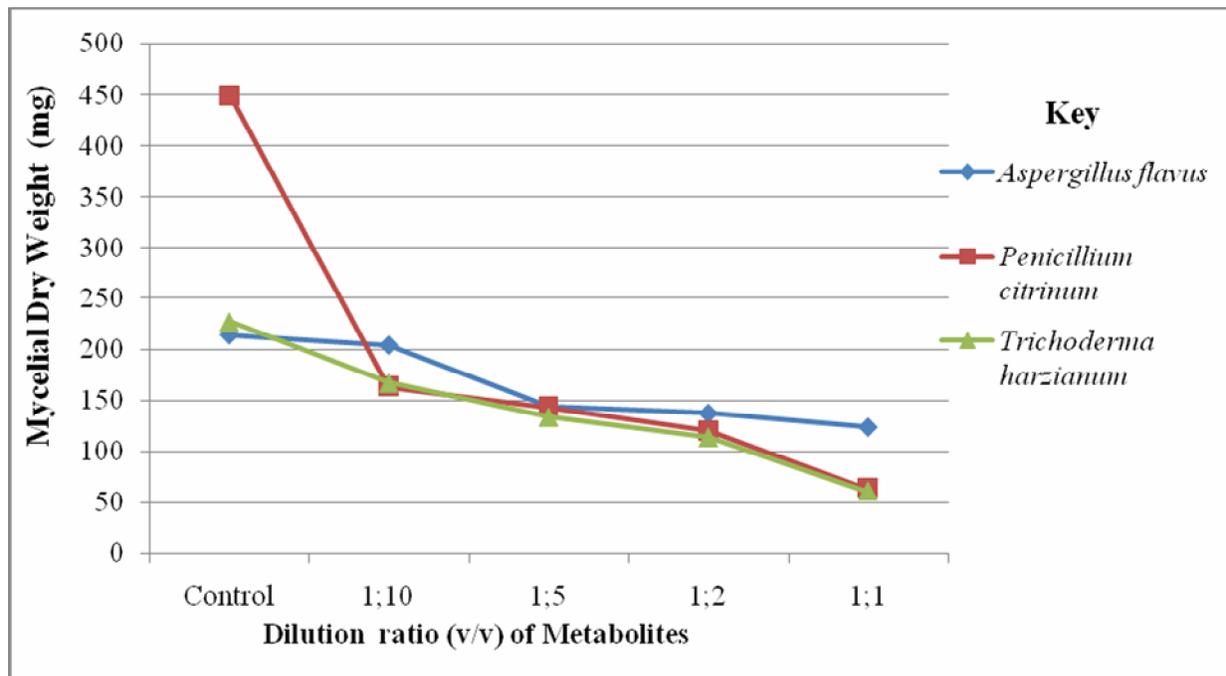


Plate.1 Radial growth of *P. eous* on Potato Dextrose Agar amended with indicated dilution of the culture metabolites of *T. harzianum* incubated at $30\pm 2^{\circ}\text{C}$ for 12 days

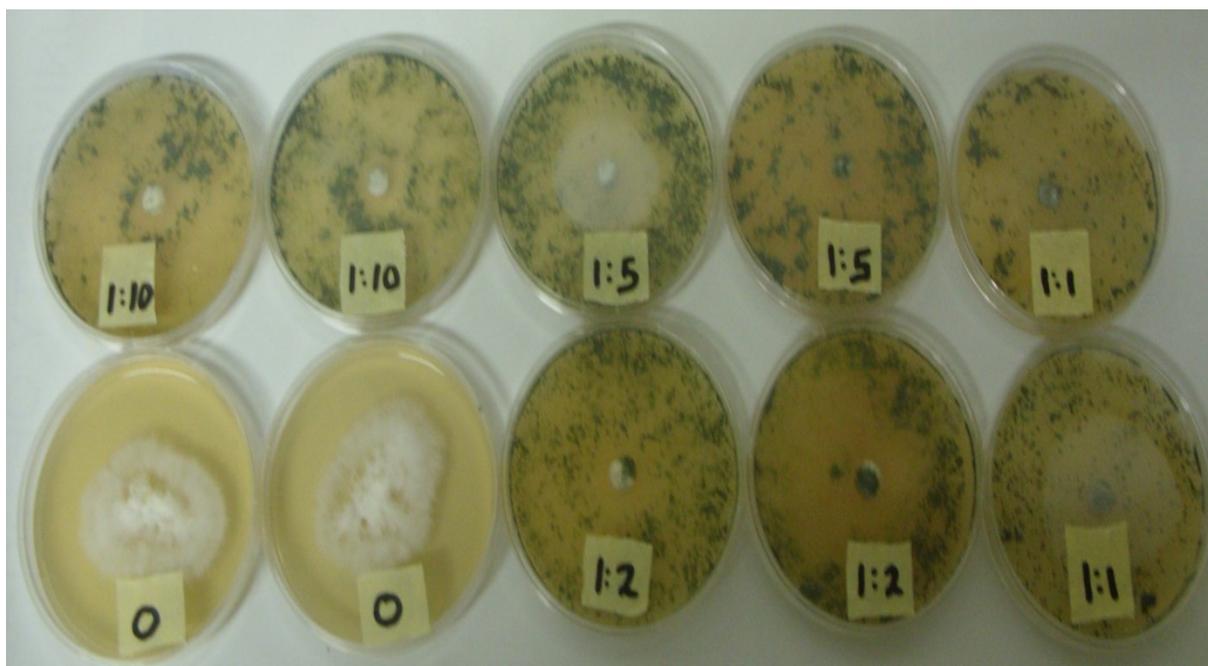


Plate.2 Radial growth of *P. eous* mycelium on Potato Dextrose Agar amended with indicated dilution of the culture metabolites of *P. citrinum* grown at $30\pm 2^{\circ}\text{C}$ for 12 days

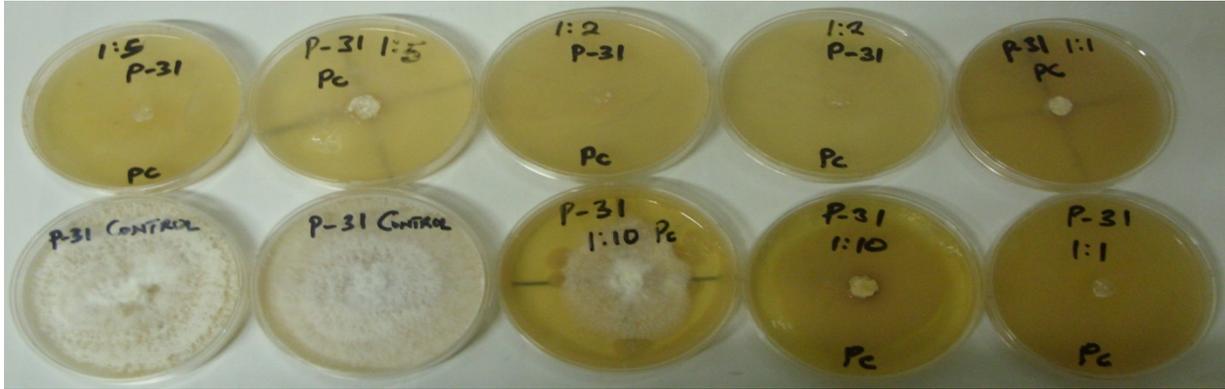


Plate.3 Radial growth of *P. ostreatus* on Potato Dextrose Agar amended with indicated dilution of the culture metabolites of *A. flavus* incubated at $30\pm 2^{\circ}\text{C}$ for 12 days

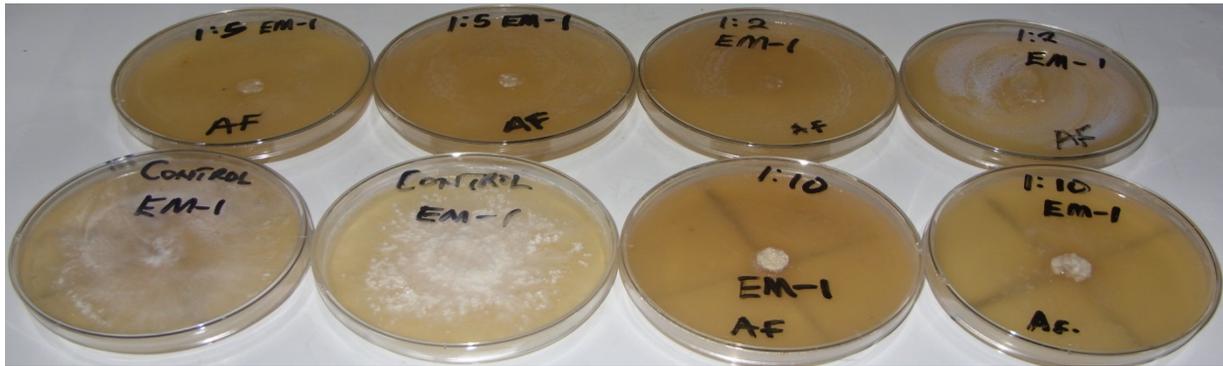


Plate.4 Influence of culture filtrate of *A. flavus* on vegetative growth of *P. ostreatus* strain EM-1 in liquid culture of Potato Dextrose Broth amended with varying concentrations (1:1 - 1:10v/v) of the culture filtrate at $30\pm 2^{\circ}\text{C}$ for 10 days (Note the reduction in vegetative growth at higher concentrations (1:1 - 1:5v/v) of the culture filtrates)



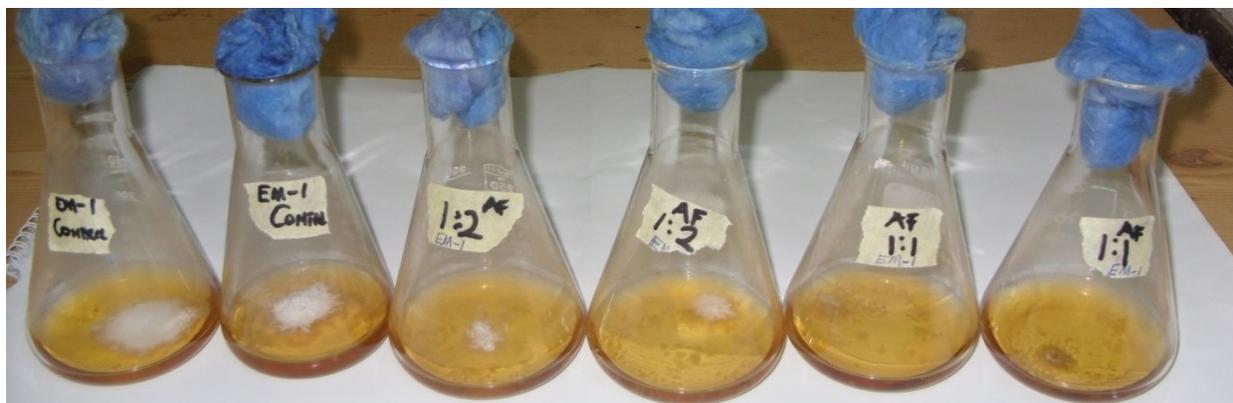
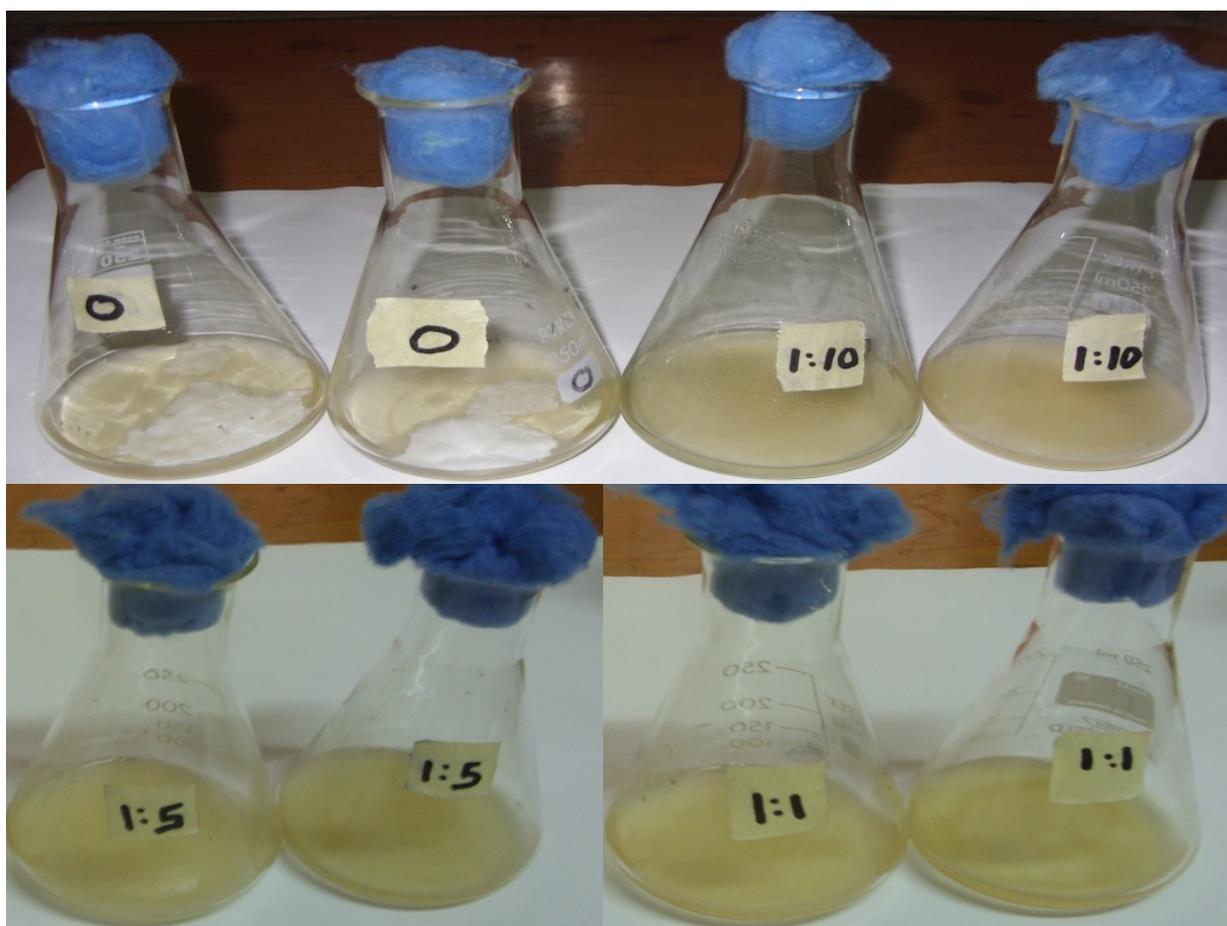


Plate.5 Influence of varying concentrations of culture filtrate of *T. harzianum* (control, 0 - 1:10v/v dilution) on vegetative growth of *P. eous* strain P-31 at 30±2°C for 10days



According to Chakraborty *et al.* (2013) and Obodai, (1992) one of the most common and destructive diseases in mushroom cultivation is the green mould caused by species of

Trichoderma, *Penicillium* and *Aspergillus*. Data from this paper agrees with this viewpoint. *T. harzianum* and *Penicillium* particularly has induced significant

qualitative and qualitative losses in yield of *Agaricus bisporus*, *Pleurotus* spp, *Auricularia*, *Calocybe indica* and *Lentinus edodes* (Seaby, 1996, 1989, 1987; Apkabay 2001; Quaicoe, 2012 and Chakraborty *et al.*, 2013). More than two decades ago (1994–1995) epidemics of *Trichoderma* green mould in the mushroom industry in Canada and USA caused losses exceeding US \$ 20 million during 3 year period (Chang and Miles, 2004). There is another health hazard posed by the detection of *A. flavus* and *P. citrinum* as contaminants of the compost. *A. flavus* produces potent carcinogenic, teratogenic and immunosuppressant mycotoxins called aflatoxins (B₁, B₂, G₁, G₂). Depending on the strain all or the B₁ type can be formed *in vitro*. However, *A. flavus* NRRL 5096 formed all four aflatoxins (Odamtten, 1987, 1986).

P. citrinum produces another mycotoxin citrinin which could be lethal to human health if picked up by the developing fruiting bodies of *P. ostreatus* and *P. eous* in the compost. There are therefore practical economic yield and health implication by the presence of *T. harzianum*, *P. citrinum* and *A. flavus* as contaminants in rice husk and straw to be used for the cultivation of *P. ostreatus* and *P. eous* which cannot be overlooked.

Recent studies by Kortei *et al.* (2014) and Kortei and Wiafe-Kwagyan (2014) compared the efficacy of moist heat sterilization and gamma irradiation treatment of several composted lignocellulose used for the cultivation of *P. ostreatus* and *P. eous*. They showed that γ -irradiation, as a treatment for sterilization of compost, including rice straw and rice husk substrate, for bioconversion to fruit bodies by *P. ostreatus* and *P. eous* is feasible and effective.

Data from this paper show that the presence of metabolites of *T. harzianum*, *P. citrinum* and *A. flavus* in rice straw and husk compost can be detrimental to the mycelial growth of *P. ostreatus* and *P. eous* and subsequently fruiting of the species in the substrate. *T. harzianum* metabolite was the most potent in its inhibitory effect on the growth of mycelium of the two oyster mushrooms followed by *A. flavus* and *P. citrinum* in decreasing order. There are therefore practical economic yield implications if contamination by particularly *T. harzianum* is allowed to reach epidemic populations in the substrate. It will also be interesting in future studies, to authenticate and elucidate the chemical nature of the lethal antibiosis agent produced by the test fungi particular by *T. harzianum*. The formulation and pasteurization of rice-based lignocellulose composts for optimum yield of *P. ostreatus* and *P. eous* under the Ghanaian tropic conditions forms the basis of a subsequent paper.

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