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Harnessing Essential Oils for in-vitro Control of *Trichoderma* sp. in Mushroom Cultivation

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Mushrooms are highly nutritious dietary items that are rich in vitamins, minerals, antioxidants, and high-quality protein. Oyster and button mushrooms are highly significant species, cultivated in diverse farms and locations. Various fungal contaminants, including *Trichoderma* sp. hindered the mycelium growth of button mushroom, hence reduces its yield. In this study, bio-management of *Trichoderma horzianum* which is a major causative agent of mushroom mold was investigated by using different essential oils viz., cinnamon, coconut, neem and rose oil with increasing concentration of 4%, 8%, 12% and 16% against *Trichoderma* sp. All of the essential oils showed significant impact, but rose oil with concentration of 16% demonstrated the highest efficacy in controlling *Trichoderma* sp against mushroom mold. Thus, it is suggested that increasing concentration of rose oil would be more effective under *in-vitro* conditions in controlling *Trichoderma horzianum*.
Keywords: Bio-management, Essential oils, *In-vitro*, Mushroom mold, *Trichoderma* sp.

INTRODUCTION

Mushrooms have been eaten since ancient times as a nutritious food, and valued for their appealing culinary characteristics. Nowadays, mushrooms have become highly valued food items due to their low calorie, carbohydrate, and fat content (Kaliyaperumal *et al.*, 2018). Mushrooms based food products possess significant amount of nutritional ingredients which are healthiest for human body (Niksic *et al.*, 2016). They provide various types of macro and micronutrients, including vitamin B complex, dietary fibers, amino acids selenium, zinc, copper and riboflavin (Das and Prakash, 2022). These components have numerous therapeutic benefits and a wide range of nutraceutical properties like healing and cleansing (Rijal *et al.*, 2021). These are the fruiting bodies of fungi with various different characteristics like epigenous as well hypogenious, visible with naked eyes so can easily be picked (Chang and Miles, 1992). Edible species of the mushroom are highly effective against some diseases including asthma and cancer, cholesterol and even insomnia (Wani *et al.*, 2010). Due to these qualities, some mushroom extracts are used to improve human health and are available as dietary supplements (Niksic *et al.*, 2016).

Mushrooms are being grown for their economic benefits more than 100 countries (Gupta *et al.*, 2018). The global mushroom industry is divided into three categories; wild mushrooms, edible mushrooms, and medicinally valuable mushrooms (Royse *et al.*, 2017). However, oyster and button mushrooms are very popular worldwide because of their edible properties (Gupta *et al.*, 2018). These essential types of mushrooms have been affected by various fungal contaminants during mycelial growth to maturity which restricting the industry of the mushroom (Rhaman *et al.*, 2021). Some notorious fungal contaminant namely, *Mycogone* spp., *Sclerotium rolfsii*, *Trichoderma* spp., *Cephalothecum roseum Coprinus* spp., *Sependonium* spp., *Lecanicillium* spp., and *Cladobotryum* spp., badly damaged mushroom crop at initial growth stages for a long time therefore mushroom industry is highly affected. These contaminants have adverse impact not only on mushroom yield but also on mushroom quality, causing overall economic harm (Amin *et al.*, 2021). Thus there is dare need of bio-management to avoid such economic losses. Care should be taken to rough the volunteer mushroom that is exhibiting Pathogen-effected symptoms, and proper sanitary

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measures can be implemented to prevent disease spread. Massive losses have been reported as a result of contaminants affecting mushrooms from substrate preparation. Such contaminants compete for mushroom spawn in the substrate, causing diseases in the mushrooms and even restricting mycellial growth (Mumpuni *et al.*, 1998). Mushroom moulds are among the most serious fungal diseases, causing significant economic losses (Biswas and Kuiry, 2013). These microbe-caused diseases damage and deteriorate mushroom quality, affecting biomass and yield and potentially leading to crop failure (Gea *et al.*, 2021). Earlier studies have indicated that various management practices, accompanied by chemical fungicides, are being widely used in controlling *Trichoderma* spp (Allaga *et al.*, 2021; Shah *et al.*, 2013). However, keeping in mind the importance of eco-friendly bio-management control is a necessary measure to be taken in order to protect the environment and human health. Thus, bio control measures having the ability to inhibit the mycelial growth of the fungi contaminants and others microbes during mushroom cultivation (Ghimire *et al*., 2021).

MATERIALS AND METHODS

A series of experiments were conducted in the department of Plant Pathology, Balochistan Agriculture College, Quetta.

***Mushroom culture and preparation of PDA media*:** Mushroom spawns of button type were purchased from online market place. Potato Dextrose Agar (PDA) culture media was prepared under laboratory condition as following standard protocol (Table 1).

Table 1. Preparation of PDA with addition of malt extract.

Ingredients	g/L DW	g/0.5L DW
Dextrose	20	10
Potato Extract	4	2
Agar	15	7.5
Malt Extract	6	3
PH	7.3 ± 2 at 25°C	

Formula adjusted standardized to suit performance parameters

***Sterilization of media*:** Sterilization of media was carried out by mixing 39g of PDA then autoclaved at 121 °C for 15 min, after this autoclaved media was cooled in laminar flow as previously described (Goss and Marr, 1963).

***Sample collection and isolation*:** Spawn of mushroom was purchased from marketplace were opened in laminar flow cabinet and picked with sterile forceps or loop to place at the center of culture media. Which was already prepared and poured at 25 plates by using similar technique and the forceps or loop is disinfected before and after each inoculation plate and incubated at 23 ± 2 °C for fifteen days so that growth of mushroom mycelium were showed on petri plates and sub cultured to get multiple pure culture and strains.

***Isolation and morphological identification of fungi*:** Isolation of *Trichoderma* sp. from infected substrates and spawn, for which wheat straw, compost and spawn were selected. After observation of contamination in these substrates it was segmented into small pieces and surface sterilized and dried on blotter paper for inoculation of PDA media poured petri plates before inoculation media along with other required material i.e., glassware forceps, loop, and scalpel were autoclaved at 121°C for 20 minutes. Laminar flow was swabbed and cooled media was poured into petri plates and allowed to solidify. After inoculation plates were incubated in incubator at 27°C for 6 days. After that, incubated petri dishes were observed for growing fungal contaminants and were identified microscopically on the slides (Fig. 1).

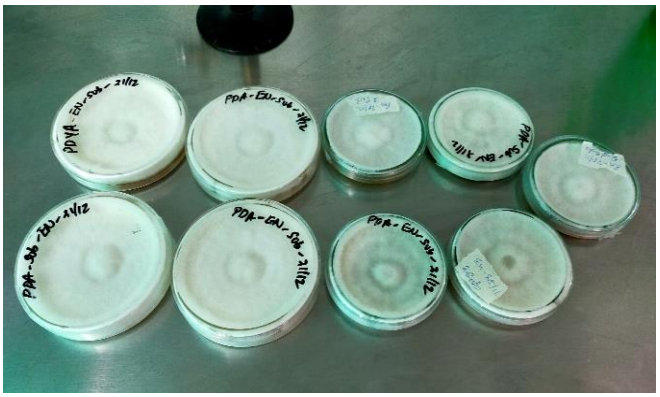

Figure 1. Pure cultures of mushroom in petri plates.

***Spawn preparation*:** Spawn was prepared using techniques of (Colavolpe *et al*., 2014) with some modifications. For spawn preparation grains of sorghum were used. The substrate sterilized and autoclaved. After sterilization the substrate was cooled at 25 °C to 30 °C before inoculation some tissue culture of button and oyster in bottle and plastic bags and incubate at 23 °C for 15 days to complete mycelium growth in the substrate.

***Seeding*:** Filled bottles with grains were placed in laminar floor, then 1-2 cubic centimeters (CC) were poured out from already prepared mycelial injections through silicon pores of the lid into the grain spawns. Secondly, the bottle lid was opened in laminar and small fresh or dried mushroom pieces were inoculated into grain bottles after proper sterilization. Inoculated jar lids were closed and sealed with Aluminum foil and placed inside incubator for two months or more depending upon the spawn growth. When white mycelium was covered in the whole jar of spawns, the jars are ready for shifting spawns to their substrate medium for further growth under controlled environment.

***Bio-management of mushroom*:** For *in-vitro* management, essential oils of cinnamon, coconut, neem and rose were

extracted manually and used to control the *Trichoderma* sp. Untreated plates were kept as control for comparison.

***Preparation of essential oils*:** The preparation of Cinnamon, Coconut, neem and rose oil was done and concentrations of 4%, 8% 12% 16% each treatment was prepared separately. The solution was prepared in 20 ml beaker, 4% concentration of each essential oil was prepared by adding 0.2 ml of extract and mix with ethanol (4.8 ml) and Tween20 of 0.25 ml and distal water (DH_2O) was 4.75 ml and total solution was 10 ml. Similarly, for 8% concentration of essential oil 0.4 ml mix ethanol 4.6 ml Tween20 0.25 ml and DH_2O 4.75 ml the total solution 10 ml. For 12 percent concentration, we added 0.6 ml of oil and mixed with ethanol 4.4 ml Tween20 0.25 ml and DH_2O 4.75 ml, the total solution was adjusted 10 ml. Likewise, for 16% concentration of essential oil 0.8 ml oil was mixed with ethanol 4.2 ml, Tween20 0.25 ml and DH_2O was 4.75 ml, final solution was 10ml.

***Mycelial growth of Trichoderma harzianum*:** Treatment of *Trichoderma harzianum* was done with all above mentioned essential oils by food poison techniques 1ml of each concentration from oil solution was added to 18 ml of PDA culture medium at 45 °C at the center of each on PDA plate after that pathogen culture was placed with the help of sterilized crock borer to check culture growth. The data was collected after every two days of interval.

RESULTS AND DISCUSSION

***Morphological identification of Trichoderma harzianum*:** The characteristics of *Trichoderma harzianum* colony appearance and sporulation pattern were examined form cultures grown on PDA media. White yellow colour media later coming green mold conidia black conidia, grey green colonies colour invisible on isolated plates at 28°C for 6 days (Fig. 2).

***Microscopic study*:** The slides were prepared to check under microscope and observed spore structures and shapes of *T. harzianum* (Fig. 2). They developed mycellium showing hyaline; branched conidiophores are produced on exposed fertile when it was grown completely on petri plate. Hyaline to green yellow oblong to globose conidia was observed with a size range 2.7~3.7 × 2.4~3.4 micro meter. Black spore masses were observed at the end of mycelium as reported in earlier study (Seaby, 1996).

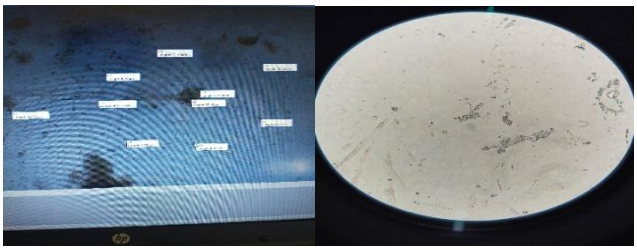

Figure 2. Morphology of pathogen spores and their sizes under microscope.

***Evaluation of cinnamon oil on mycelial growth of Trichoderma harzianum*:** Data given in Table 2 was compared with control (C) having highest growth rates 71.32 mm due to non-application of the essential oils and 0% reduction of the colony growth. For the treatment of cinnamon oil at 4% concentration, the highest total growth rate of the pathogen was 69.07 mm in petri plate and the least reduction in the colony growth percent was 3.15 Second concentration was 8% that exhibited 63.32 mm of growth and the reduction in the colony growth percent was 11.22. Third concentration with 12% indicated total colony growth of 60.25 mm and the reduction in the colony growth percent was 15.52 The least total growth of pathogen colony recorded at 16% concentration dosage was 30.68 mm and the highest decrease in the colony growth percent was 56.98.

Fungistatic and fungicidal activity values of the various essential oils have been evaluated for their efficacy in the experiment for their fungal inhibiting growth ability against *Trichoderma harzianum* among which most effective antifungal agent includes cinnamon and thyme at concentration of 0.125 and 0.0625 µl/ml respectively. *T. viride*, *P. pinophilum* and *P. chrysogenum* mycellial growth reduced 40.7%, 40.9% and 50% respectively with the use of clary sage oil (Angelini, *et al*., 2006). The results show significant difference in the linear colony growth of *Trichoderma harzianum* in which P value was less than 0.05 with application of Cinnamon oil concentrations.

***In vitro evaluation of Coconut oil on mycelial growth Trichoderma harzianum*:** All the data in Table 3 was

Table 2. *In-vitro* efficacy of Cinnamon on colony growth of *Trichoderma harzianum*.

Treatment	Dose	Linerar colony growth (cm)			Total growth	Reduction in colony growth (RCG)	Reduction percentage
		2nd Day	4th Day	6th Day		RCG = C – TCG	RCG (100)/C
	4%	22.40	51.02	69.07	69.07	2.25	3.15
Cinnamone	8%	18.20	45.02	63.32	63.32	8.00	11.22
	12%	11.40	42.22	60.25	60.25	11.07	15.52
	16%	9.25	19.98	30.68	30.68	40.64	56.98
Control (C)		26.18	53.02	71.32	71.32	0.00	0.00

Table 5. *In-vitro* efficacy of Rose on colony growth of *Trichoderma harzianum.*

Treatment	Dose	Linerar colony growth (cm)			Total growth	Reduction in colony growth (RCG)	Reduction percentage
		2nd Day	4th Day	6th Day		RCG = C – TCG	RCG (100)/C
	4%	5.65	46.13	51.50	51.50	21.05	29.01
Rose	8%	5.42	26.33	33.63	33.63	38.92	53.65
	12%	4.32	17.05	27.33	27.33	45.22	62.33
	16%	3.92	14.68	19.02	19.02	53.53	73.79
Control (C)		10.32	62.18	72.55	72.55	0.00	0.00

compared with control (C) having highest growth rates 81.53 mm due to non-application of the essential oils and 0% reduction of the colony growth. For the treatment of Coconut oil at 4% concentration, the highest total growth rate of the pathogen was 58.68 mm in petri plate and the least reduction in the colony growth percent was 28.03. Second concentration was 8% that exhibited 53.62 mm of growth and the reduction in the colony growth percent was 34.23. Third concentration with 12% indicated total colony growth of 38.45 mm and the reduction in the colony growth percent was 52.84. The least total growth of pathogen colony recorded at 16% concentration dosage was 32.54 mm and the highest decrease in the colony growth percent was 60.09.

At 5% concentration of coconut oil *T. harzianum* mycellial growth was recorded minimum up to 9cm and in control plates it was recorded 62cm (Hassan, *et al.*, 2022). The results show significant difference in the linear colony growth of *Trichoderma harzianum* in which P value was less than 0.05 with application of Coconut oil concentrations.

***In Vitro* evaluation of Neem oil on mycelial growth *Trichoderma harzianum*:** All the data in Table 4 was compared with control (C) having highest growth rates 68.20 mm due to non-application of the essential oils and 0% reduction of the colony growth. For the treatment of Neem oil at 4% concentration, the highest total growth rate of the pathogen was 61.17 mm in petri plate and the least reduction in the colony growth percent was 10.31. Second concentration was 8% that exhibited 54.57 mm of growth and the reduction in the colony growth percent was 19.99. Third concentration with 12% indicated total colony growth of 50.32 mm and the reduction in the colony growth percent was 26.22. The least total growth of pathogen colony recorded at 16% concentration dosage was 43.97 mm and the highest decrease in the colony growth percent was 35.53.

Antifungal activity of Neem, oil, clove, ajwain and others were tested by agar diffusion plate method against fungi viz. *Trichoderma* sp. 106-1, and *Trichoderma viridi* 108-1 etc. which showed significant results against the fungi mentioned. Remarkable reduction was observe by all the essential oil among which most effective growth inhibition were observed in neem oil (0.5%) treated plates upto 80.05 ± 0.06% against *Trichoderma* sp. 108-1 and 75.65±0.01% against *Trichoderma* sp. 106-1 (Hussain, *et al.*, 2013). The results show significant difference in the linear colony growth of *Trichoderma Harzianum* in which P value was less than 0.05 with application of Neem oil concentrations.

***In Vitro* evaluation of Rose oil on mycelial growth *Trichoderma harzianum*:** All the data in Table 5 was compared with control (C) having highest growth rates 72.55 mm due to non-application of the essential oils and 0% reduction of the colony growth. For the treatment of Rose oil at 4% concentration, the highest total growth rate of the pathogen was 51.50 mm in petri plate and the least reduction in the colony growth percent was 29.01. Second concentration was 8% that exhibited 33.63 mm of growth and the reduction in the colony growth percent was 53.65. Third concentration with 12% indicated total colony growth of 27.33 mm and the reduction in the colony growth percent was 62.33. The least total growth of pathogen colony recorded at 16% concentration dosage was 19.02 mm and the highest decrease in the colony growth percent was 73.79.

Mycellial inhibition were evaluated at various concentrations % of essential oils viz., lemongrass oil, Rose oil and thyme oil through in in-vitro experiment, among all rose oil inhibited

Table 4. *In-vitro* efficacy of Neem on colony growth of *Trichoderma harzianum.*

Treatment	Dose	Linerar colony growth (cm)			Total growth	Reduction in colony growth (RCG)	Reduction percentage
		2nd Day	4th Day	6th Day		RCG = C – TCG	RCG (100)/C
	4%	9.85	53.63	61.17	61.17	7.03	10.31
Neem	8%	9.23	43.32	54.57	54.57	13.63	19.99
	12%	9.05	42.97	50.32	50.32	17.88	26.22
	16%	6.50	29.92	43.97	43.97	24.23	35.53
Control (C)		10.95	58.20	68.20	68.20	0.00	0.00

the mycellial growth of *Trichoderma harzianum* at 1.0% concentration by 71.7% thyme and lemongrass showed effiecient results at 0.5% but rose showed highest fungal inhibition (Abdel-Kader *at el.,* 2013). The results show significant difference in the linear colony growth of *Trichoderma harzianum* in which P value was less than 0.05 with application of Rose oil concentrations.

***Conclusion*:** It is concluded that during the in-vitro management *Trichoderma harzianum* can be controlled through essential oil viz., Rose, Coconut, Cinnamon and Neem oil out of which Rose Oils has effective results against the mycellial growth of pathogen; *Trichoderma harzianum*.

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***Ethical statement*:**This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

***Consent to participate*:** Gohar Khan and Muhammad Waris conceived the idea and prepared manuscript.

***Consent for publication*:** All authors are giving the consent to publish this research article in Phytopathogenomics and Disease Control.

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