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Effects of *Trichoderma viride* and *Aspergillus* on the growth and Nutritions of *Pleurotus ostreatus*

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Abstract—It is evident that the Oyster mushroom readily enjoys wheat straw as a better substrate in comparison to the other substrates used for cultivation. The present study conducted to check the nutritional status of the the Oyster mushroom harvested from the bag contaminated by the *Trichoderma viride*. The result showed the unexpected and unusual positive role of *Trichoderma viride* for the growth of *Pleurotus ostreatus* and biochemical changes in the test mushroom. This is justified by the biochemical analysis of mushroom fruiting bodies harvested from the bags contaminated by the *T.viride* and mushroom fruiting bodies harvested from the non-contaminated bags.The analytical result showed a lowering of glucose and sucrose concentration where protein and total dietary fibre was found to be increased; moreover, fructose and total fat have also increased when compared to mushrooms harvested from non-contaminated bags.

Keywords—Dietary fiber, Protein; Trichoderma sp; Pleurotusostreatus

I. INTRODUCTION

Pleurotus mushrooms are simplest and easily cultivable on the agric residue available on the agric farm; different substrate types have been used for the cultivations to increase the yield (Dehariya & Vyas, 2020; Hoa et al.,2015; Satpal et al., 2017).Worldwide Oyster mushroom cultivations are in practice, according to the suitability of sources and the cost-effective viability of the substrate for cultivations. The common substrate for Oyster mushroom cultivation used is wheat straw, sawdust, paddy straw, corn cob, sugarcane baggage, ground nutshell, etc. (Dehariya & Vyas, 2013; Jain & Vyas, 2003; Vyas et al., 2003). However,wheat straw or supplemented wheat straw was found to be the best substrate; wheat straw contains alarge number of lignin and cellulose (Chaubey et al.,2010; Draganova et al., 2018). *Pleurotus* produced the enzyme system to degrade the lignocellulosic components of the substrates and made them available for the mushroom for their metabolism, which makes the mushroom a rich source of protein, and dietary fibre, vitamins, and minerals (Hoa et al., 2015). Though many substrates have been tested for the oyster mushroom growth, keeping in the view to enhance the yield of the mushroom (Dehariya & Vyas, 2020; Hall, Yun, & Amicucci, 2003; Hoa et al., 2015; Mohamed et al., 2014; Naraian & Bharti, 2017) however very few attempts have been made to enhance the biochemical substances which are important for therapeutical purpose. Undoubtedly, some attempt has also been made to manipulate the genome of mushrooms for the better synthesis of biomolecules particularly (Santoyo et al., 2008).

Like the plant disease,the mushrooms have also suffered from the disease caused by fungi, bacteria, viruses, and abiotic factors; the most common contaminant of the *Pleurotus* is *Trichoderma sp* and the *Aspergillus sp* causing mold diseases in mushrooms; it has been reported that the contamination by these fungi leads to the loss of mushroom crops worldwide. However, the bags infected by the *Aspergillus sp* results in complete loss of the mushroom crop, but the bags contaminated by the *Trichoderma sp* have 30%-50% loss subject to infectivity rate.

Aspergillus releases mycotoxin that might inhibit the growth of the other fungi, moreover many species of *Aspergillus* have got complex enzyme system that efficiently dissociates the complex structure of wheat straw and produces soluble saccharides (Sarkar & Aikat, 2014) that may act as a feedback inhibition mechanism for the enzyme released by the *P.ostreatus* to accumulate necessary nutrition for growth and as well as exhibit toxic effects on the mycelium of Oyster mushrooms.

The present study is an accidental experiment where, under natural conditions, *Trichoderma* contaminated bags showed a good yield of Oyster mushroom and valuable changes in the number of biomolecules. In contrast, the bags contaminated by the *Aspergillus niger* showed complete loss of mushroom productions.

According to Colavolpe et al. (2014), *Trichoderma*, a fungal weed, does not hamper the growth of *P.ostreatus* rather, it promotes the growth of the mushroom; it is deduced that enough carbohydrate is available for *P. ostreatus* growth. It was presumed that enzymatic batteries of *P.ostreatus* help it to avail proper substrate. However, excessive growth of *Trichoderma* reduces the *P.ostreatus* growth; on the other hand, 20-30% contamination does not affect the *P.ostreatus* growth, instead, it induces a positive change in the nutritional composition of the mushroom. This unusual finding compels us to investigate the results.

II. MATERIALS AND METHODS

The culture of *Pleurotus ostreatus* was used for spawn production was obtained by the Lab of mushroom Biology, Department of Botany, Dr.Harisingh Gour Vishwavidyalaya, Sagar India.

Spawn production

Spawn was prepared in half-litre capacity wide-mouthed glass bottles. The grains were cleaned to remove any broken,

shrivelled grains handpicking of undesired grains. After this, the grains were soaked overnight in clean water and then washed.They were boiled in water for 15 minutes, ensuring that grains should not split but remain slightly hard after boiling. Excess water was removed and cooled to 25-30^{0}C. The cooled grains were then mixed with 1.2 percent commercial-grade gypsum ($CaSO_4$) and 0.3 percent calcium carbonate ($CaCO_3$). Gypsum prevents the sticking of wheat grains together, and calcium carbonate maintains the pH of 5.5 - 7.5. The grains were filled in the bottles, plugged in non-absorbent cotton covered with butter paper, and then sterilized at 121^{0}C (15 lbs pressure) for 2 hours on two consecutive days. Sterilized bottles were taken out from the autoclave while still hot and shaken to avoid grains clumping. Sterilized bottles were inoculated from the pre-maintained culture of *P.ostreatus* by 9 mm disc in an individual bottle. The spawn bottles were incubated without shaking at 24±1^{0}C in the B.O.D incubator.

***Substrate preparation*:**

Wheat straw was used for this experiment. Wheat straw @ ten kilograms (10kg) of wheat straw/100liters of water soaked in a plastic container, solution of Carbendazim (8gm/100liter water) + Formalin (120ml/100liter water) was added for chemical sterilization of straw for 18 hrs, the container was covered with a polyethylene sheet to prevent the evaporation of the formalin. After that, the straw was taken out from the solution and kept for 2-3 hours to drain excess water (Dehariya & Vyas, 2020).

Spawning

Spawning was done under aseptic conditions. Spawn of *P.ostreatus* was mixed in Wheat straw (substrate) @ 4 percent per kg on a dry weight basis and 3kg substrate (containing 60-75% moisture) filled in each polythene bag (22×12″) a total of 30 bags was inoculated and made 8-10 holes in each bags for aeration. After spawning, bags were kept in the mushroom house under dark conditions, and observations were recorded.

Identification of Fungal Trichoderma and Aspergillus.

The identification of the *Trichoderma* sp and the *Aspergillus* sp was confirmed based on microscopic (Fig-3), morphological, and cultural conditions.

***Measurement of Average cap diameter, Average stipe length, and weight of mushroom*.**

The measurement of the average cap diameter and stipe length was based on the methods adopted by (Dehariya & Vyas, 2013a; Kortei et al., 2018; Yang, Guo, & Wan, 2013).

Stipe length= Length of cap base to end of the stalk.

$$ACD = \frac{L_d + S_d}{2}$$

Where, ACD= average cap diameter, L_d longest cap diameter, S_d= shortest cap diameter

The weight of the mushroom fruiting body of the first harvest was measured using an electronic balance, and the data were recorded in Table-2.

In vitro study to check the compatibility of Pleurotus with Aspergillus and Trichoderma

To check the compatibility of *P.ostreatus* with the *A.niger* and *T.viride*, a dual culture technique was used; in brief-PDA medium was made, and the plate was poured with 16 ml of PDA in plate after the solidification of the medium, 4mm cube of *P.ostreatus* culture was placed on half side of the plate and another half side was inoculated by the 4 mm cube cut of *A.niger* culture, the same was done for the *T.viride.*

Determination of Sugar content, total Dietary fiber, and Protein content in fruitbodies.

***Sugar content**: (fructose, Glucose, Maltose, and lactose)*

The method described by Silva et al. (2018) with some modifications have been used, a 100 g L^{-1}sugars standard stock solution (SSS) of fructose, Glucose, Maltose, and lactose (Sigma- Aldrich, >99.5%), was prepared by dissolving in 25 mL of water and working standard obtained 0.005,0.01,0.015,0.02 and 0.025 by further dilution of the stock solution with 12% v/v ethanol. Neocuproine, 1.041 g dissolved in 5 mL of ethanol and was added to an aqueous solution containing 0.4 g of copper sulfate pentahydrate (Sigma-Aldrich, 99%) and the mixture (Neocuproine complex) was diluted to 50 ml with water, and a working standard was obtained to .005,0.01,0.015,0.02 and 0.025 concentration with further dilutions. A 1.0 mg/ml sodium hydroxide stock solution was prepared by dissolving 4.0 g of NaOH (Rankem) in 100 mL water; the working standard was obtained by dilution of the stock into a concentration of .005,0.01,0.015,0.02 and 0.025.Now each concentration of SSS, Neocuproine complex, and NaOH was added in a volumetric ratio of 1:1:1, and a standard curve was plotted.

Sample preparations

The powder samples of *T.viride* contaminated bag and sample of non-contaminated bag's mushroom were dissolved in (12 % V/V) of ethanol and were continuously stirred for a duration of 2-3 hours. And then extracts were filtered, and the absorbance was taken in Systronics 22002 UV-Vis Double beam spectrophotometer at 460nm.

Estimation of Sucrose

Sucrose determination was done by the Anthrone method described by David T. Plummer (1990) with some modifications.From the standard stock solution of 200 ppm glucose working concentrations 0.1; 0.2; 0.3; 0.4;0.5;0.6, and 0.7, was made and brought to 1 ml by dilutions in a test tube, a blank solution containing 1 ml of distilled water was considered. Into each standard glucose solution and the blank, 5 ml of anthrone reagent was added and closed, vortex, and shaken until evenly distributed. The test tube was heated above 100ºC water bath for 12 minutes. Solutions were transferred to cuvette after cooling, and absorbance was recorded using a UV-Vis spectrophotometer with a wavelength of 630 nm, and a standard curve was plotted. Following the same procedure, the mushroom sample was run to read the absorbance to estimate sucrose using the standard curve.

Estimation of Total Dietary fibre

The determination of dietary fibre was conducted on dry basis methods developed by the FSSAI, (2015)equation given below have been used in the determination of dietary fibre.

$$\text{Df (on dry basis)} = \frac{\text{IW-FW}}{\text{IW}} \text{ X } 100$$

Where, Df = Dietary fibre; IW= Initial weight; FW= Final weight

***Estimation of Protein content*:**

The estimation of the total protein was done by the Kjeldahl Method described by the BIS (2000),with slight modification.

$$\text{W n1} = \frac{(V_0\text{-}V_1) \text{ x } C_1 \text{ x } M}{m}$$

Where,

W n1 = Total Nitrogen content in gm/Kg

V_0 = Volume in milliliters of Sodium Hydroxide solution required to test for the blank

V_1 = Volume in milliliters of Sodium Hydroxide solution required to test for the sample

M= Molar mass of Nitrogen

m = mass of the sample taken for the test

W_P = Crude Protein = Wn x 6.25

C_1= Concentration of Na OH

Table-1: Effects of contaminants on the growth of *P.ostreatus*

Conditions of cultivated mushroom in the mushroom house.		Spawn run of *Pleurotus* (Days)	Pinhead appearance of *Pleurotus*(Days)
Contaminated bags	*Aspergillus niger* *Trichoderma.viride*	No runs. Patches of *Pleurotus on day 19th*	No growth 57(first harvest)
Non-contamiant ed bags	Mycelium of *Pleurotus*	19	24(first harvest)

III. RESULTS

Spawn run

In the mushroom house, temperature (22°-26°C) and relative humidity (80-90 %) was maintained during the spawn run. Humidity was maintained by water spraying three times a day. After the complete run of spawn in the straw, it becomes a compact mass, bags were opened for sporophores formation by removing polythene and kept in cropping room, but the case was not same for all the bags some of the bags were invaded by the *T.viride and A.niger*, the bags which were infected by the *T.viride* and *A.niger* were identified based on a morphological character under the microscope, and kept aside for observation. At the time of sporophores formation, the windows were kept open for 1–2 hrs to provide fresh air, release CO_2, and maintain the relative humidity at 80-90 percent inside the crop room.

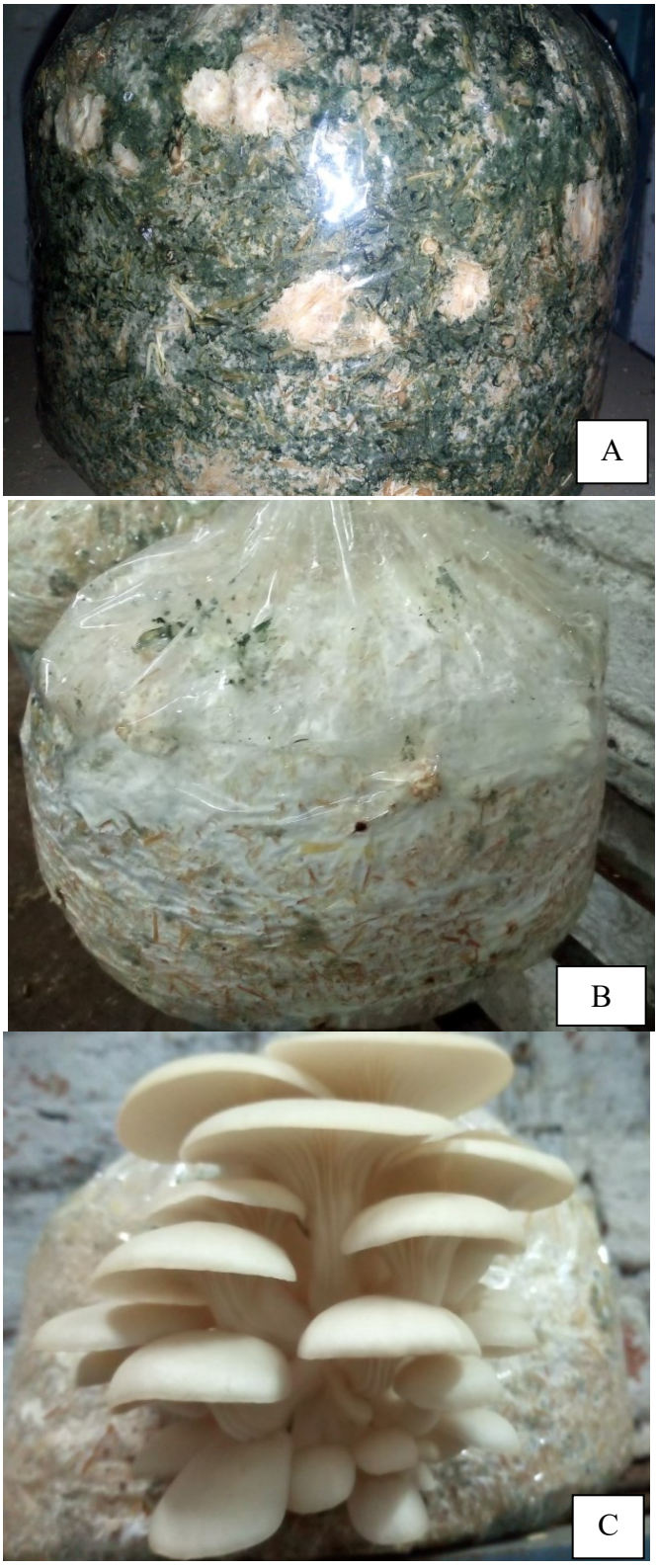
Table-2: Table showing measurement of cap diameter, stipe length weight of fruiting bodies of the mushroom harvested from *Trichoderma* contaminated bag and non-contaminated bags.

Conditions of the bags	AverageCap diameter (cm)	Averagestipe length (cm)	Weight of first harvested fruiting bodies (gm)
Trichoderma contaminated bags	13.4	2.1	280
Non-contaminated bags	12.5	2.4	300

Sporocarp production

After the spawn run, a compact substrate (wheat straw) was kept in the crop room for the sporophores production. The fruiting bodies were started to appear in 6-8 days (20-22 days for spawn run and 6-8 days needed for fruiting bodies).The bags which were contaminated by the *T.viride* are kept aside; no fruiting body has been seen in these bags; only the patches of *Pleurotus* mycelium seem to be grown but were dominated by the mycelium of *T.viride* while the bags which were contaminated by the *A.niger* completely colonized the bags.The fruiting bodies of non-contaminated bags were harvested 3-4 days after pinhead initiation. The total time for cropping up to the third flush was 60-70 days. The watering of the crop was done with a mist sprayer to maintain the mushroom house moisture (80-90 %); the contaminated bags were also given the same treatment. Adequate ventilation in the crop room was provided by opening the doors and windows at night for a short time. The fruiting bodies were protected from direct sunlight, but some diffused light (2500-3000 Lux) was allowed to induce fruiting body formation. The crop room floor and wall were sprayed with 0.1 percent Malathion to protect it from insect infestation and to prevent the fungal infection; sprays of Carbendazim 0.02 percent were also given.Harvestingthe sporophores of *P.ostreatus* were done after maturity. After the first harvesting, the bags were remained without irrigation for three days and then again irrigated after pinhead initiation. The exact process was followed afterthe second harvesting. Since the *T.viride* is characterized by a short life cycle and high rate of growth, the mycelium after spore production seems to be dormant, probably due to the exhaust of specific nutrients available for the *Trichoderma*, the resurgence of the *P.ostreatus* was seen in the bags contaminated by the *Trichoderma* and within few days the whole bags completely run over by the *P.ostreatus* mycelium, but the bag that contaminated by the *A.niger* has not shown any such growth as shown by the *Trichoderma* contaminated bags. The first harvest was done after 57th days from the bags contaminated by *T.viride*, while the

first harvest on the non-contaminated bags was done after the 24thdays of spawning (see table-1). The harvested fruiting body was freeze-dried and kept for further study.



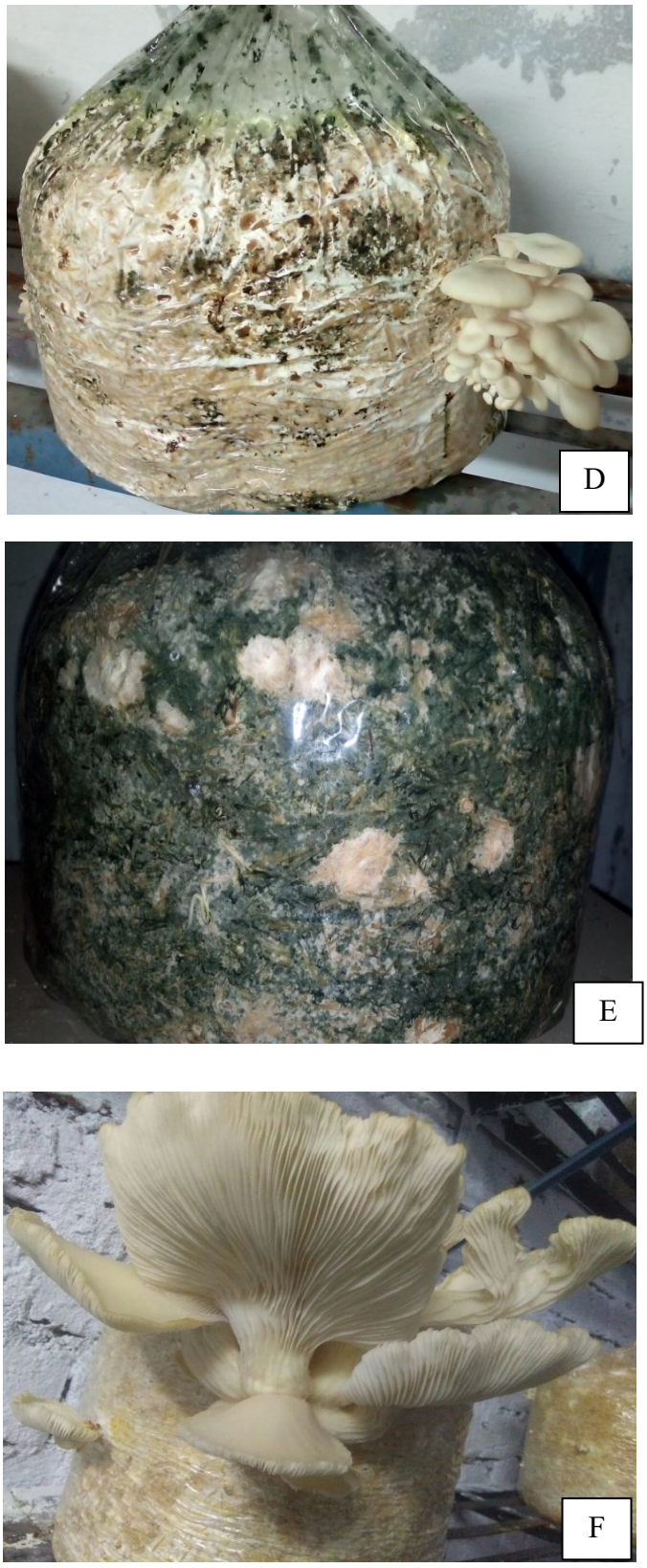


Figure-1: Contaminated bags by *T.viride*, A-Initial Invasion of *T.viride* on mushroom; B- a resurgence of Mushroom Mycelium; C,D Fruiting body of oyster on contaminated bags by*T.viride*; E- *A.niger* contaminated bag; F-Non-contaminated bag.

Invitro study

The in-vitro study conducted by using dual culture techniques showed that the plate cultured with *A.niger* +*Pleurotus* showed complete inhibition of the *Pleurotus* mycelium and the plate cultured with *T.viride* + *Pleurotus* showed co-existence of mycelium each fungus, see in figure-2.

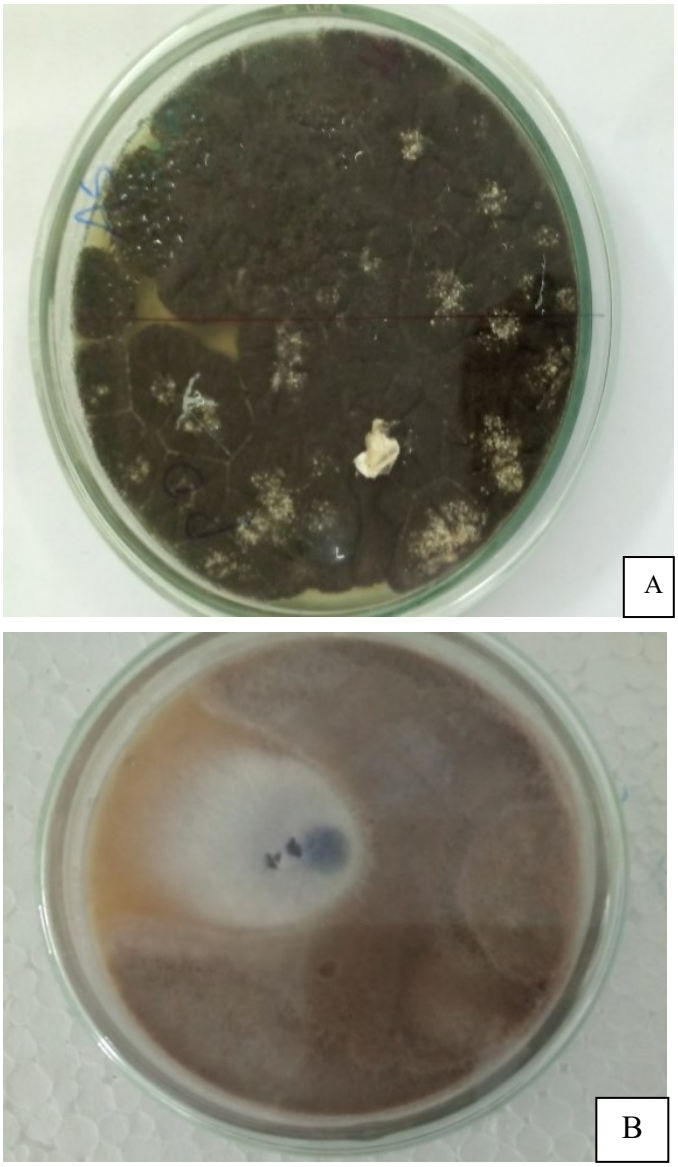


Figure-2: A- *A.niger* inhibited plate, arrow showing *Pleurotus* culture disc; B- Showing Co-existence of *T.viride* and *P.ostreatus.*

IV. Discussion

It was seen in the study that the glucose and sucrose content of the *P.ostreatus* contaminated by the *T.viride* has lowered (1.8mg/100gm and 1.46mg.100gm of the sample, respectively) compared to uncontaminated bags (2.3mg/100gm and 2.37mg100gm of the sample respectively), but this was opposite in case of the fructose see table-3, this may be because the substrate rich in carbohydrate which promotes *T.viride* growth, it was also noted that the bags at the initial stage of spawn run if contaminated by *T.viride* helps profuse growth of *T.viride* (fig-1A), however, this biotic stress on the *P.ostreatus* induces production of laccase enzyme for self-defence (Colavolpe et al., 2014). *Trichoderma* sp secretes various cellulolytic enzymes to degrade the cellulosic content of the substrates (Gordillo-Fuenzalida et al., 2019), in a spectrometry study carried by Gordillo-Fuenzalida et al., (2019) revealed that *Trichoderma* sp produces extracellular protein/enzyme endoglucanases, exoglucanases, and β-glucosidases;also produces a higher amount of protease activities on the wheat substrate. However, some of the *Trichoderma* sp also produces chymotrypsin (proteolytic enzyme) activities (Hatvani, 2008), due to the presence of *Pleurotus* mycelium on the wheat substrate, the *Trichoderma* sp uses different enzymatic strategies to adapt to the circumstances that exist during *Pleurotus* cultivation. Hence, *Trichoderma* has high competition for the substrate than the *Pleurotus* (Hatvani, 2008) and efficiently used source of carbon available on wheat substrates. This may be the reason for the lower glucose and sucrose content in the mushroom contaminated by the *T.viride* compared to the non-contaminated bags.

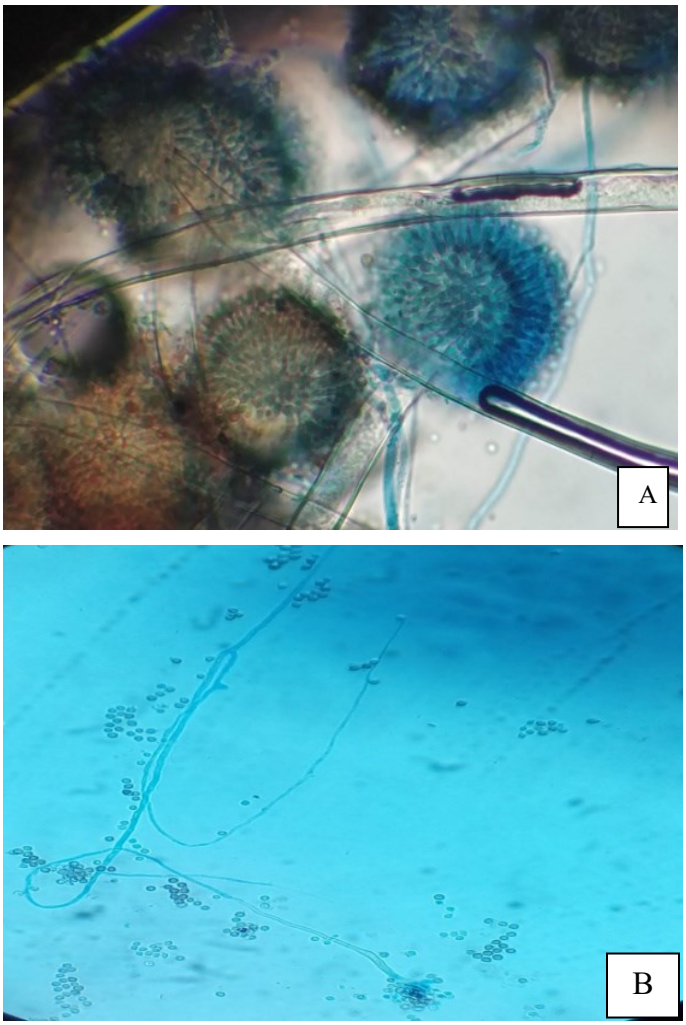


Figure-3: A-Microscopic structure of *A. niger* (under 40X); B-Microscopic structure of *T.viride* (under 10X).

P. ostreatus do not have lactose and maltose, as our findings showed. We found enhanced protein content in the mushroom fruiting body harvested from the *T.viride* contaminated bags compared to the non-contaminated mushroom bags; the reason behind this may be due to the enzyme complex released by the *T.viride* under the competition with *P.ostreatus* for carbohydrates, which makes the complex polysaccharides of wheat straw into simpler sugar forms and other derivatives that further soon after the completion of the *T.viride* life cycle utilizedbythe *P.ostreatus*, that might have enhanced the protein content of the *T.viride* contaminated bags, it has also been reported that the some of the fungi grow on low nitrogen content and may fix nitrogen in the substrate (Wiafe-Kwagyan et al., 2015) in this case, *T.viride* may have fixed the nitrogen which was later utilized by the mushroom; moreover, there has been a report of nitrogen-fixing bacteria to be associated mushroom substrate. Mushrooms are also known for their dietary fibre; in our finding, we show an increment of total dietary fibre(TDF) in the mushroom harvested from contaminated bags by the *T.viride*; *Pleurotus* contain mostly higher non-digested carbohydrates, i.e. dietary fibre including oligosaccharides and the polysaccharides of the cell wall of the fungus(e.g. Chitin, beta-glucans, and mannans) while the digestible fibre is less than 10% in the mushrooms, these non-digestible fibres help in preventing colon disease and haemorrhoids in humans (Hariram et al.,2014), the fact that how the dietary fibre increases in the mushroom harvested from the *T.viride* contaminated bags lies in the enzyme complex and the mechanism of degradation of the wheat straw. Whereas the bag contaminated by the *A.niger* does not show the growth of the *P.ostreatus* and has completely inhibited the mycelium of *P.ostreatus,* as seen in fig-1E.

Table-3: The nutritional value of Mushrooms in the sample from non-contaminated bags and contaminated bags.

Nutrients	Mushroom from non-contaminated bags	Mushroom from contaminated bags
Glucose	2.3 mg/100 gm	1.8 mg/100 gm
Sucrose	2.3736 mg/100 gm	1.4616 mg/100 gm
Fructose	0.2mg/100 gm	0.8 mg/100 gm
Lactose	Absent	Absent
Maltose	Absent	Absent
Total Dietary Fibre	43.33 %	47.3%
Protein	25.59 gm/kg	28.938 gm/kg

In dual culture methods with *A.niger* and the *P.ostreatus* showed complete inhibition of the *Pleurotus* mycelium. Whereas the dual culture with *T.viride* and the *P.ostreatus* shows co-existence in the culture plate (Jayalal & Adikaram, 2007), this observation confirms that the *A.niger* have intense antagonistic activities whereas *T.viride* competes for the nutrition and space only.

Moreover, it can be concluded that the bag contaminated by the *A.niger* had no mycelia growth of *P.ostreatus* and showed complete inhibition of the *P.ostreatus* growth. Whereas the bag contaminated by *T.viride* delayed the mycelium run of *P.ostreatus,* and as a consequence, the mycelium of *Pleurotus ostreatus* resurged entirely after the 57th day, the delayed resurgence of the *P.ostreatus* mycelium may be due to the higher content of nitrogen in substrates as the *T.viride* favoured its growth in high rich of carbon, which resulted into the reduction of carbon from the substrate which results in the increment of nitrogen ratio to the carbon in the wheat straw. Higher nitrogen content inhibits the growth of the mushroom in excessive amounts (Yang et al., 2013). The average cap diameter and stipe length of *P.ostreatus* that were harvested from the contaminated bag by *T.viride* were more as compared to the non-contaminated bags. However, the weight of the fruiting bodies of the first harvest was greater in the non-contaminated bags than the contaminated bags by *T.viride*.This may be happened due to the lower C/N ratio in the contaminated bags by the *T.viride* as lowered C/N ratio favoured the fruiting bodies growth(Kortei et al., 2018; Yang et al., 2013). This accidental finding concludes that the *T.viride* contaminated bags initially lowered the *P.ostreatus* growth. However, after the resurgence of *P.ostreatus* mycelium,the fruiting bodies appeared, which,after nutritional analysis, showed lower glucose and sucrose concentration, whereas protein and total dietary fibre were increased.

Moreover, fructose and total fat have also increased compared to mushrooms harvested from non-contaminated bags. Hence it can be concluded that *T.viride* does not harm the growth of *P. ostreatus*; instead, in the presence of the *T.viride,* the nutritional composition of the *P.ostreatus* changes, which enhances the edibility of the *P.ostreatus*. However, the infested bags by *T.viride* delayed the fruiting body's production of *P.ostreatus.*

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Author contribution:

All the authors contributed equally to the concepts of the study; Chandan Singh, Neelam Chaudhary, Priya Pathak and Apeksha Rathi prepared the methodology and prepared sample for the analysis, Poonam Dehariya and Deepak Vyas has revised the manuscript and conceptualized the conclusion sections and rectified the manuscript, firstly draft by the Chandan Singh. The final manuscript of the previous draft was read and approved by all the authors.

Conflict of interest:

Authors declare no conflict of interest

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