MICROBIAL CONTAMINANTS IN OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*) CULTIVATION THEIR MANAGEMENT AND ROLE OF METEOROLOGICAL FACTORS

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ABSTRACT

Survey revealed the occurrence of seven contaminants in mushroom beds and out of which *Trichoderma harzianum, Penicillium notatum, Sclerotium rolfsii* and *Coprinus* spp. were found to be most dominant fungal contaminants and occurrence was high during June and July (28.4 & 35.8 %) causing maximum loss to mushroom yield. The incidence of contaminants were minimum during January (2.87%) and maximum during the month of June (32.8 %). A good harvest of mushroom (105% BE) was obtained during the month of October. A range of average maximum temperature (24.63 – 33.18 ^{o}C), minimum temperature (9.40 -25.51 ^{o}C) and average relative humidity (68.90 -85.27%) was found most appropriate for the cultivation of oyster mushroom in this region. Among the botanicals tested for management of competitors moulds, *Azadirachta indica* (neem) showed its supremacy and exhibited maximum inhibitory effect (54.1 to 71.6 %) against *Aspergillus* spp., *Trichoderma* spp., *Coprinus* spp., and *Penicillium* spp. and was found to be less effective against *Sclerotium rolfsii in vitro* followed by extracts of *Pongamia pinnata* (42.4 to 61.3%). A range of 35.3 to 62.4% reduction in inky caps (*Coprinus sp.*) and 26.3 to 68.4% in green moulds (*Trichoderma* spp) were recorded with different phyto-extracts. The botanicals except *Acacia nilotica* reduced the incidence of competitor moulds (18.18 to 70.91%) in mushroom beds which increase the yield up to 21.3 %. The study will provide the idea of appropriate cultivation time as well as provide an alternative method of surface sterilization.

Keywords: oyster mushroom, environmental factors, contaminants, plant extract, botanicals

INTRODUCTION

Oyster mushroom (*Pleurotus* spp.) belonging to class Basidiomycetes and family Agaricaceae is popularly known as 'dhingri' in India. The popularity of oyster mushroom has been increasing due to its ease of cultivation, high yield potential and high nutritional value [1, 2]. Oyster mushroom help to remove the toxicity produces by the agro wastes [3-5]. The use of fungicides for controlling the competitor moulds and diseases in oyster mushroom cultivation is very common in India. The hazardous effects of chemicals in human health and environmental aspect are known. Apart from these problems continuous usage of same chemicals may lead towards pest's resistance. Studies on various aspects of fungal contaminants and diseases of *Pleurotus* spp. were undertaken by various workers [6-8] and they reported *Trichoderma harzianum*, *Aspergillus* spp., *Penicillium* spp., *Monilia sitophila, Stemonitis* spp. and *Coprinus* spp. were the major contaminants of *Pleurotus* spp. These species become prevalent in *Pleurotus* cultures if the substrate has not been uniformly or properly pasteurized. Among these contaminants, *Trichoderma harzianum* was reported to be the most damaging one, competing aggressively with the mycelium of *P. pulmonarius* and *P. ostreatus in- vitro* and reducing the production surface from 30 to 50% [9]. While, *Aspergillus niger*, *Coprinus* sp, *Penicillium* sp and *Sclerotium rolfsii* were the most predominant fungal contaminant of mushroom beds of *P. florida* [10]. Spilman [11] recognized *Trichoderma* as green mould on the production bed of oyster mushroom. *Trichoderma, Aspergillus* and *Rhizopus* on oyster mushroom bed were predominant microorganisms and especially occurrence of these was severe in summer and spring seasons than autumn and winter [12]. Considering the above, an experiment was conducted to develop a suitable management practice against the competitor moulds of *Pleurotus ostreatus* in an eco- friendly manner under the agro-ecological condition of lateritic belt of West Bengal.

MATERIALS AND METHODS

Survey for Natural Incidence of Competitor Moulds: Five home scale mushroom farms of Bolpur and its adjacent areas were surveyed every month from January 2010 to December 2011 for the occurrence of contamination in mushroom beds of oyster mushroom (*P. ostreatus)*. The incidence of different competitor moulds were recorded. Infected mushroom bags were tagged and the contaminated microflora were identified. Total number of infected beds were counted from each farm. In addition, five mushroom beds were raised in Ballabpur, Bolpur farmar's cropping room in first week of every month and allowed for spawning and yield. Month wise average data on three important weather factors viz., temperature, relative humidity and rainfall were recorded. Spawn of oyster mushroom (*P. ostreatus)* was supplied from the laboratory to the progressive farmers for cultivation. Paddy straw was taken as substrate and chemical sterilization technique [13] was followed in cultivation. Container system of cultivation (polythene bags) was followed in all experiments. Samples of various diseases and competitor fungi were collected on a regular basis from the cropping room and subsequently, *in vitro* studies have been carried out in laboratory.

Preparation of Beds: Chopped paddy straw was soaked into a solution containing the requisite amount of sterilizing agent for 16-18 hours. Thorough spawning @ 4% by wet weight basis was followed. The spawned substrate was filled in polypropylene bags (45x30 cm). A unit of 2 kg of dry straw was used for each treatment, which was equally distributed in four bags representing each as a replication. The moisture content of the straw at the time of spawning was kept around 72-75%. The filled bags were incubated in a dark room at a temperature ranging between 24-30 ^{o}C, where 90% relative humidity was maintained till the spawn run was complete. When the straw is fully covered with milky white mycelium in the bag, it is regarded as complete spawn run, then the bags were cut open and compacted mass of aggregated straw called, as "bed" was ready for cropping. The beds were hanged by nylon string at a distance of 60 cm. Harvesting was done when the small primordial converted into a full grown sporophore.

Isolation and Purification of Competitor Moulds: Competitor moulds fungi were collected from the damaged beds in sterilized petriplates with the help of a sterile forceps and thereafter transferred into PDA plates under *in vitro* conditions. Inoculated PDA plates were incubated at 27 ^{o}C ($\pm$ 2 ^{o}C) for 3 to 4 days. A single colony was isolated from the PDA plate and again transferred to PDA plates for obtaining the pure culture. All the pure cultures were kept in refrigerator at 4 ^{o}C for preservation.

Preparation of Photo-Extracts: For preparation of phyto-extracts, 100 gram plant products were collected, washed in tap water, air dried and homogenized with equal amount of distilled water (100 ml) by crashing them with electric grinder machine. The extract was filtered through double-layered muslin cloth and centrifuged at 4000 rpm, for 10 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper which was considered as standard solution.

***In vitro* study:** Different eco-friendly botanicals i.e. *Azadirachta indica,Lantana camera, Pongamia pinnata,Acacia nilotica,Clerodendron indicum,Eucalyptus hybrid,Datura metal, Cassia tora* and chemicals (carbendazim 75 ppm + formalin 500 ppm) were evaluated individually against competitor moulds and oyster mushroom (*P. ostreatus*). Poisoned food technique [14] (Grove and Moore, 1962) was carried out to evaluate the inhibitory effect of fungal mycelia. For one competitor mould, 4 ml of each plant extract (standard solution) was incorporated in 100 ml of potato dextrose agar medium (PDA) and autoclaved for 20 minutes at 1.41 kg/ cm^2 pressure. The molten media were poured into four sterilized glass petriplates (90 mm) considering each as a replication. After solidification of the agar plates were inoculated with 5 mm diameter mycelial cut from 6 day old culture of competitor moulds i.e. *Aspergillus* spp., *Trichoderma* spp., *Coprinus* spp., *Penicillium* spp and *Sclerotium rolfsii* and *P. ostreatus*. The petriplate without any treatment served as control. The plates were incubated at room temperature till the complete growth observed in control plates. The mycelial inhibition was calculated as [(dc-dt)/dc] x 100 where dc was dia of control and dt is dia in the treatment.

***In vivo* study:** Phyto-extracts and chemicals were further evaluated *in vivo* to see their inhibitory effect against the major contaminants, i.e. *Trichoderma* sp., *Aspergillus* sp., *Coprinus* sp., *Sclerotium rolfsii* and *Penicillium* spp. Paddy straw was dipped into the solution containing appropriate concentration of phyto-extracts and chemicals separately for 16-18

h and spawning was done @ 4% wet weight basis. The polythene bags were cut open when colonization was completed. Untreated paddy straw was used as control. Data on different parameters were collected on a regular basis during the cropping. Percent contamination index was calculated for the test and control beds depend upon the following scale

Grade 0: 0% – incidence of contaminants
Grade 1: >0 – 20% coverage by the contaminants
Grade 2: >20 – 40% coverage by the contaminants
Grade 3: >40 – 60% coverage by the contaminants
Grade 4: >60 – 80% coverage by the contaminants
Grade 5: >80 – 100% coverage by the contaminants

Per cent contamination index = (sum of the total scores/maximum rating) x total number of observations x 100

RESULTS

Role of Meteorological Factors: The effect of fluctuations in climatic factors on the incidence of competitor moulds and yield of oyster mushroom was studied and the data obtained are presented in Table 1. Survey revealed the occurrence of seven contaminants i.e. *T. harzianum*, *P. notatum*, *A. niger*, *Coprinus* spp., *Mucor* sp., *Rhizopus* sp., and *S. rolfsii* in mushroom beds and out of which *T. harzianum*, *P. notatum*, *S. rolfsii* and *Coprinus* spp. were found to be the most dominant fungal contaminants. The incidence of the contaminants were minimum during the month of January (2.875 %) and it increased considerably with the fluctuating climatic conditions and reached its peak during the month of June (32.8 %). Thereafter, a decline trend in contamination % was noticed in this region. Minimum range of contamination (2.95 to 5.2%) was observed during the period from January to February and again from October to December, when maximum biological efficiency 105% was obtained. A range of average maximum temperature (24.63–33.18 °C), minimum temperature (9.40 -25.51 °C) and average relative humidity (68.90 -85.27%) was found most appropriate for the cultivation of oyster mushroom in this region (Fig. 1). Significant positive correlations were obtained between average rainfall (1.00**) and minimum temperature (0.695*) and incidence of microbial contaminants in mushroom beds. However, relative humidity in cropping room was the most deciding factor of yield of mushroom (0.779**). High R^2 values of (1.00**) and (0.916**)

Table 1. Relationship between meteorological factors,incidence of microbial contaminants and yield of oyster mushroom

Cropping month	Mean temperature (°C)		Mean RH % m.m.	Total Rainfall	Contamination %			Yield (Kg/bed) in Ballabpur farm	Biological Efficiency%
	Max.	Min.			Five Farmer's farm (avg.)	Ballabpur farm	Average		
January	24.63	9.40	68.90	3.1	2.80	2.95	2.87	0.815	81.5
Febuary	29.62	14.72	82.65	16.1	6.19	5.2	5.69	0.895	89.5
March	36.88	20.80	65.34	6.9	8.10	6.4	7.25	0.776	77.6
April	40.28	25.55	52.9	54.9	16.31	14.9	15.60	0.598	59.8
May	36.0	25.41	46.1	109.5	21.30	18.5	19.9	0.420	42
June	36.29	25.86	49.4	172.8	35.8	32.8	34.3	0.371	37.1
July	33.91	26.33	81.94	267.2	30.2	28.4	29.3	0.595	59.5
August	33.72	26.38	85.73	111.0	24.2	21.4	22.8	0.786	78.6
September	33.18	25.51	85.27	177.8	13.4	12.2	12.8	0.883	88.3
October	33.04	23.27	82.80	57.3	5.60	4.7	5.15	0.985	98.5
November	31.24	18.79	78.21	11.9	4.30	3.8	4.05	1.050	105
December	25.28	12.67	77.13	33.1	3.50	3.90	3.7	0.930	93

indicated 100 % contribution of all meteorological factors on the incidence of contaminants and about 91.6% contribution of meteorological factors and microbial contaminants respectively towards the yield of *P. ostreatus*, Table 2. and 3.

Table 2. Correlation between meteorological factors, incidence of microbial contaminants and biological efficiency of oyster mushroom

Sl.No	Meteorological factors	"r" Values	
		Incidence of microbial contaminants (%)	Biological Efficiency (%)
1	Maximum Temperature	0.349	-0.568
2	Minimum Temperature	0.695*	-0.5120
3	Average Relative Humidity	0.028	0.779**
4	Rainfall	1.000**	0-.530

* Significant at 5% level ** Significant at 5% level

Sl.No.	Multiple correlation between	R^2 Value
1.	Y^1 and X^1, X^2, X^3, X^4	+1.00**
2.	Y^2 and X^1, X^2, X^3, X^4, X^5	+.0.916**

**** = Significant at 1% level * = Significant at 5% level**

Where : X^1 = Maximum temperature X^2 = Minimum temperature
X^3 = Average relative humidity X^4 = Rainfall
X^5 = Average contamination %
Y^1 = Average contamination %
Y^2 = Biological efficiency%

***In vitro* study:** The extent of inhibition of mycelium growth of *P. ostreatus* and different competitor moulds varied considerably with different botanicals and chemicals used (Table 4). Significant differences were obtained among all the treatments. Chemical treatment (bavistin 75 ppm + formalin 500 ppm) proved its superiority among all the treatments and found to be most effective in inhibiting the mycelial growth of five contaminants (65.4 to 86.6 %). Among the botanicals, *A. indica* (neem) showed maximum inhibitory effect (54.1 to 71.6 %) against the growth of four competitor moulds fungi i.e. *Aspergillus niger*, *Trichoderma viride*, *Coprinus* spp. and *Penicillium* sp., and was found less effective against the mycelium growth of *P. ostreatus* (4.4%). This was followed by extracts of *Pongamia pinnata* (karanja) 42.4 to 61.3% (mould fungi) and 6.7% (*P. ostreatus*) and *Clerodendron indicum* (clerodendron) which inhibited 40.0 to 53.8% and 8.9% mycelium growth of mould fungi and *P. ostreatus* respectively. Among the five contaminants, *Sclerotium rolfsii* was reported to be more resistant against most of the botanicals used. However, the growth of the same fungi was reduced considerably with the extract of *P. pinnata* (53.3%) and *Eucalyptus hybrida* (43.3%).The extracts of *Acacia nilotica* showed minimum inhibitory effect (18.9 to 35.3%) against the contaminants tested and also exhibited adverse effect on the growth of mushroom mycelium (25.6%) as given in Fig. 2.

***In vivo* study:** The response of eight plant extracts and chemicals used as surface sterilizing agent for cultivation of *P. ostreatus* are presented in Table 4. Data indicated the supremacy of chemical treatment over the botanicals used. An increase of 35.20 % in biological efficiency and 81.36% reduction in the incidence of competitor moulds were observed from chemically treated substrate. Similar trends were also noticed with the botanicals *in vivo* ,where maximum yield of mushroom (95.10 % B.E.) was obtained from the substrate treated with *A. indica* which checked 70.91% incidence of competitor moulds in beds. This was followed by *P. pinnata* 92.20% (B.E.) and 61.36% (reduction in mould incidence) and *Clerodendron indicum* treated substrate 89.00% and 56.82% respectively (Fig. 3).

The substrate treated with chemicals (bavistin 75 ppm + formalin 500 ppm) has taken minimum period (14 days) for completing the spawn run where, no moulds attack has been noticed. The extract of *A. indica* showed excellent response towards the growth of mushroom mycelia and completed the spawn run within 16 days. It also reduced the incidence level of competitor moulds (4.1% PCI). However, no correlation exist between the treatments in terms of average weight of sporophores.

Table 3. Multiple correlation between meteorological factors, incidence of microbial contaminants and biological efficiency of oyster mushroom

S.N.	Treatments	Dose	Radial growth of mycelium and percentage growth inhibition 8 days after inoculation{mycelium growth in (mm) and growth inhibition in (%)}												Sem± Treatment mean	CD at 5% (mycelium growth)
			Pleurotus ostreatus		*Coprinus sp.*		Aspergillus niger		*Penicillium sp.*		*Sclerotium rolfsii*		*Trichoderma* spp			
			mm	%	mm	%	mm	%	mm	%	mm	%	mm	%		
1	*Azadirachta indica*	4%	86.0	4.4	29.3	62.4	31.2	54.1	22.4	71.6	68.0	24.4	27.2	68.4	0.899	2.671
2	*Lantana camera*	4%	73.0	18.9	54.0	30.8	*45.0*	33.8	50.0	36.7	62.0	31.1	61.0	29.1	1.067	3.170
3	*Pongamia pinnata*	4%	84.0	6.7	30.2	61.3	35.0	48.5	43.0	45.6	42.0	53.3	49.5	42.4	0.853	2.536
4	*Acacia nilotica*	4%	67.0	25.6	50.5	35.3	52.0	23.5	54.0	31.6	73.0	18.9	63.4	26.3	1.106	3.286
5	*Clerodendron indicum*	4%	82.0	8.9	36.0	53.8	36.5	46.3	38.0	51.9	54.0	40.0	51.0	40.7	1.02	3.032
6	*Eucalyptus hybrida*	4%	80.0	11.1	39.0	50.0	42.0	38.2	33.0	58.2	51.0	43.3	46.0	46.5	1.027	3.052
7	*Datura metal*		71.0	21.1	37.0	52.6	35.0	48.5	46.0	41.8	57.0	36.7	55.0	36.0	1.130	3.358
8	*Cassia tora*	4%	75.0	16.7	41.0	47.4	49.0	27.9	58.0	26.6	64.0	28.9	59.0	31.4	0.971	2.887
9	Bavistin + Formalin l	75ppm+ 500ppm	83.0	7.8	15.0	80.8	23.5	65.4	15.5	80.4	25.0	72.2	11.5	86.6	0.824	2.450
10	Control		90.0	0.0	78.0	0.0	68.0	0.0	79.0	0.0	90.0	0.0	86.0	0.0	0.726	2.158
	Sem± Treatment mean		1.125		0.887		0.965		1.037		0.953		0.826			
	CD at 5%		3.250		2.563		2.787		2.997		2.754		2.387			

Table 4. *In vivo*evaluation of different phyto-extracts and chemicals against major competitor moulds of oyster mushroom

Sl No.	Treatment	Dose	Average yield from 500 g substrate (g)	Biological Efficiency %	Average weight of sporophore(g)	Days to emergence of Pinhead	% increase/ decrease in B.E.	% incidence of CM	% (+) /(-) in incidence of CM	Remarks
1	*Azadirachta indica*	4.0%	475.5	95.10	6.9	16	21.30	4.1	-70.91	-
2	*Lantana camera*	4.0%	412	82.40	6.4	26	5.10	7	-31.82	*Cs, An, Sr.*
3	*Pongamia pinnata*	4.0%	461	92.20	6.85	17	17.60	5	-61.36	-
4	*Acacia nilotica*	4.0%	354	70.80	5.32	26	-9.69	24	+9.09	*Cs, An, Ps, Sr*
5	*Clerodendron indicum*	4.0%	445	89.00	6.75	19	13.52	6	-56.82	_
6	*Eucalyptus hybrida*	4.0%	425	85.00	6.2	21	8.42	7.8	-36.36	*Sr, Cs*
7	*Datura metal*	4.0%	436	87.20	6.1	20	11.22	6.5	-45.45	_
8	*Cassia tora*	4.0%	402	80.40	5.9	23	2.55	8.5	-18.18	*Sr, Tr*
9	Bavistin +Formalin	75 ppm+ 500 ppm	530	106.00	7.3	14	35.20	0	-81.36	
10	Control		392	78.40	5.8	24	0.00	22	0.00	*Cs, Ps, Sr,Tr*
	SE (treatment mean)			4.251	0.850	0.235	0.695	0.683		
	CD at 5%			12.279	2.455	0.679	2.007	1.973*		

Cs = Coprinus sp., *As = Aspergillus niger, Ps = Penicillium* sp., *Sr = Sclerotium rolfsii, Tr = Trichoderma spp., CM= Competitor moulds*

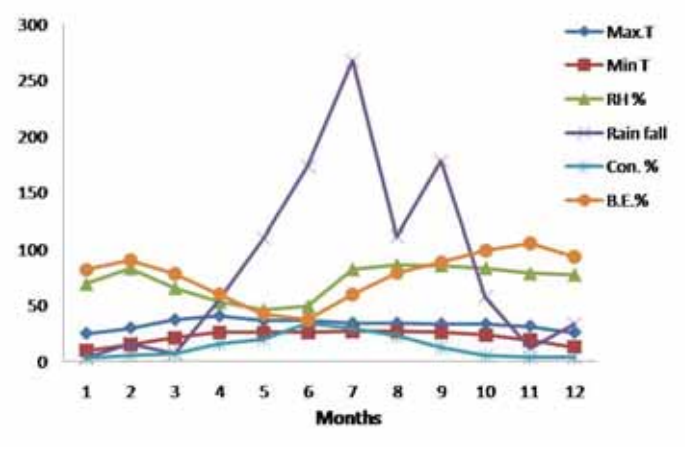

Figure 1. Relationship between meteorological factors, incidence of microbial contaminants and yield of oyster mushroom

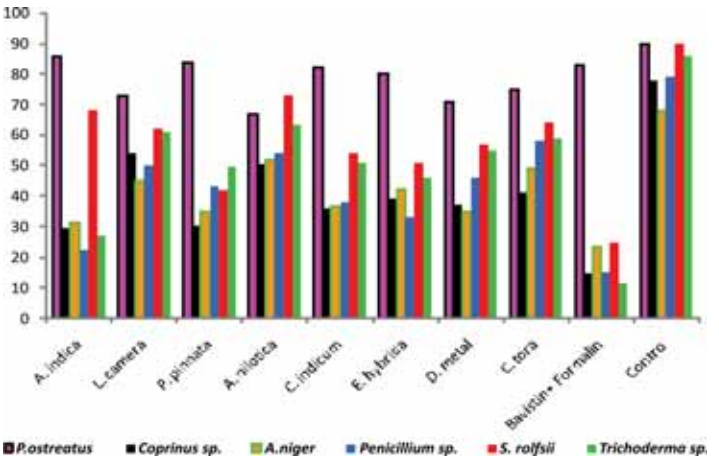

Figure 2. *In vitro* evaluation of botanicals against competitor mould fungi

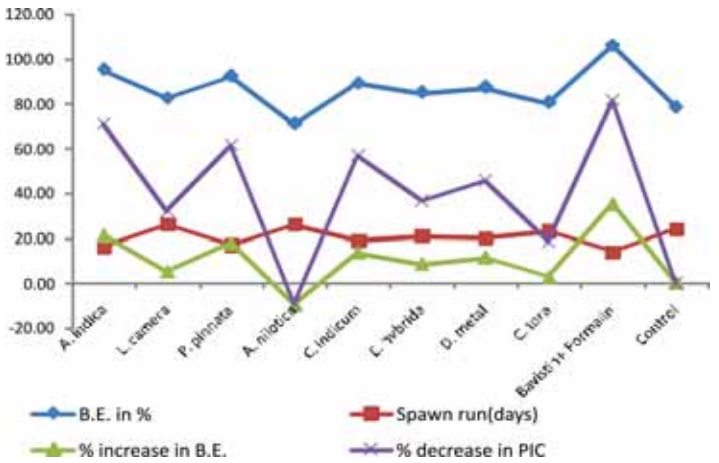

Figure 3. *In vivo* evaluation of different botanicals against competitor mould fungi and B.E.

DISCUSSION

The optimal temperature for the spread of the mycelium or vegetative growth of *Pleurotus* spp. is around 25-28 °C [15] and for fruiting body formation the temperature requirement is nearly 2 to 4 °C less than that. If there is too cold then the mycelial growth is to be arrested and at more than optimum temperature there is the risk of mould and bacterial contamination on the production beds which leads to destruction of the mushroom mycelia. In present investigation, heavy rain coupled with elevated minimum temperature (>25 °C) increased the contamination in beds was probably due to the decreasing of oxygen supply and increase in CO_2 concentration in the mushroom house or growing bags which may reduce the growth rate of oyster mushroom mycelia. Different concentration of carbendazim (bavistin) and it's combination with formaldehyde (formalin) were evaluated against the major contaminants of *P. sajorcaju*, *P. flabellatus* and *P. citrinipileatus* [13,16] and they reported complete inhibition of the mould fungi under in vitro and/ or in vivo. Complete inhibition of most of the competitor moulds of oyster mushroom was obtained with the application of 50 ppm benomyl + 100 ppm thiram [17,18]. In the present study the inhibition patterns of carbendazim 37.5 ppm + formalin 500 ppm against the competitor moulds *in vitro* further support the findings of Jain & Vyas [19]. Carbendazim show high affinity for tubulin protein in fungi, a heterodimetric protein with subunits as alternating helices of α and β tubulin, which forms an essential part of fungal cytoskeleton as well as are active in spindle formation. Thus it primarily acted upon cell and nuclear division of fungi which inhibited the mould growth completely on the beds and hence increased the biological efficiency of mushroom (106 %). Phyto-extract of *A. indica* (neem) showed maximum inhibition against the competitor moulds was due to the presence of

antifungal and antibacterial molecules azadirachtin, limonoids and terpinoids [20,21]. Leaf extract of *A. indica* having antifungal properties against *A. parasiticus* an aflatoxin producer [22] it's azadirachtin, and meliantriol etc. interfered with the release of ammonium during the early stages of fruiting body formation of *Coprinus* spp [23]. The above mentioned facts can be attributed to the higher biological efficiency of *P. ostreatus*. Extract of *P. pinnata* (karanj) leaf and oil contain karanjin, oleic acid, karanjic acid and their three esters, karanj ketone and its oxime derivaties. All these compounds have outstanding antifungal activity [24]. The karanj based products exhibited outstanding antifungal activity against the soil-borne phytophagous fungus like *S. rolfsii* (Sacc.) [25]. Excellent response of *P. pinnata* leaf extract against *S. rolfsii* was probably due to the detrimental effect of one or more above mentioned antifungal molecules, which not only minimize the growth of fungal contaminants but also contributed to spawn run period (17 days) and biological efficiency (92.20%). Good response of leaf extractof *E. hybrida* against *S. rolfsii* was probably due to the effect of 2', 6'-dihydroxy-3'-methyl-4'-methoxy-dihydrochalcone, eucalyptin and 8-desmethyl-eucalyptin which have strong antifungal and antibacterial activity [26, 27].

CONCLUSION

Microbial contamination of oyster mushroom bed is one of the major hindrance in increased yield of *Pleurotus* spp. under the agro-ecological condition of undulating red and lateritic belt of West Bengal, which was maximum during the rainy season. Plant extract have shown considerable promises as an effective alternatives for minimizing the infection of competitor moulds and diseases of oyster mushroom under the agro-ecological condition of lateritic belt of West Bengal,India. Leaf extract of *A. indica* (neem) showed maximum inhibitory effect against the growth competitor moulds which increased the biological efficiency of oyster mushroom (*P. ostreatus*) up to the tune of 95.10 % as against 106 0%, obtained through popular chemical method (carbendazim 37.5 ppm + formalin 500 ppm) commonly practiced in this region. *A. indica* (neem) and *P. pinnata* (karanj) are the two important perennial trees having tremendous medicinal values and found abundantly in this area. Leaf extract of these plants can be used successfully for surface sterilization of substrate during oyster mushroom cultivation which not only protect the environment and human health from hazardous effects of chemicals but also minimize the cost of cultivation.

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