STRAIN IMPROVEMENT OF OYSTER MUSHROOMS– *PLEUROTUS CYSTIDIOSUS* O.K.MILL AND *PLEUROTUS OPUNTIAE* (DURIEU AND LEV.) SACC.

by

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(2015-21-008)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"STRAIN IMPROVEMENT OF OYSTER MUSHROOMS–*PLEUROTUS CYSTIDIOSUS* O.K.MILL AND *PLEUROTUS OPUNTIAE* (DURIEU AND LEV.) SACC."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

Date: 04-10-2018 **Krishnapriya. P.J**

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
lbs	Pound per square inch
mm	Milli meter
^{0}C	Degree Celsius
CD	Critical difference
cm	Centimetre
et al.	And other co workers
g	Gram
h	Hours
i.e.	That is
ml	Milli litre
kg	Kilo gram
min.	Minutes
mg	Milli gram
Sl. No.	Serial number
sp. or spp.	Species (Singular and plural)
viz.	Namely
pH	Negative logarithm of hydrogen ions
µm	Micron meter
µl	Microlitre
ppm	parts per million
wt.	weight
BE	Biological efficiency
temp./T.	temperature
RH	Relative humidity
CRD	Completely Randomized Design
L	Litre

Introduction

1. INTRODUCTION

Mushroom is a "macrofungus with a distinctive fruiting body which can be either epigeous or hypogeous and large enough to be seen with the naked eye and to be picked by hand" (Chang and Miles, 1992). Hawksworth (2001) estimated that 1.4 lakh species of the estimated 1.5 million fungi in nature produced mushroom fruiting bodies of sufficient size and structure. Of these, over 3,000 species from more than 30 genera are regarded as edible mushrooms, with only 100 species grown experimentally and 60 species cultivated commercially and economically. Mushrooms have been cultivated and used as nutritious food since ancient times for their nutritional value and flavour. Nutritional value is attributed to low amount of calories and rich supply of carbohydrates, essential amino acids, fibre, vitamins and minerals (Keneni and Kebede, 2014). Mushroom has several therapeutic properties including anti-cancer, antagonistic properties against HIV-1 and numerous other diseases (Beelman *et al.,* 2003).

China tops the list of world's largest producer of mushrooms with an annual production of about 5 million tons and is the biggest producer of *Lentinula*, *Pleurotus*, *Auricularia*, *Volvariella*, *Flammulina* and *Tremella*. *Lentinus edodes* is the world's leading cultivated edible mushroom with about 22 per cent of the world's supply. *Lentinula* and four other genera (*Pleurotus*, *Auricularia*, *Agaricus*, and *Flammulina*) account for 85 per cent of the world's total supply of cultivated edible mushrooms. Oyster mushroom is cultivated worldwide, especially in South East Asia, India, Europe and Africa. They can be cultivated under both temperate and tropical climatic conditions and harvested all over the year.

Out of 3000 edible mushrooms known, about 283 species are known to be found in India (Singh, 1999). However, three types of edible mushrooms are mainly cultivated in India on commercial basis *viz*., white button mushroom (*Agaricus* spp.), oyster or dhingri mushroom (*Pleurotus* spp.) and paddy straw

mushrooms (*Volvarcella* spp.) (Chakraborti and Sikdar, 2008), with the white button mushroom contributing about 85 per cent of the country's production (Wakchaure *et al.*, 2010).

Oyster mushroom belongs to Class Basidiomycetes and Family Agaricaceae (Carla *et al.*, 2013). It is rich in proteins, vitamins, crude fibre and has been recommended for its lovastatin properties, to patients with cholesterol related ailments (Cheung, 2010). It is the easiest and least expensive commercial mushrooms which can readily convert crop residues to food protein (Caglarirmak, 2007). In Kerala, around 20 species of *Pleurotus* are being cultivated, of which *P. florida*, *P. eous*, *P. sajor caju*, *P. djamor* are the widely appreciated species. The occurrence of two new species of *Pleurotus viz. cystidiosus* and *opuntiae* in Kerala have been reported by Geetha (2011; 2015). *P. cystidiosus* also known as abalonus/brown, Miller's/Maple oyster mushroom has the longest shelf life, minimum spore discharge, long duration, with bigger size, thick, fleshy and buff coloured sporocarps. Coremial and non-coremial isolates are also reported in *P. cystidiosus*, based on the presence/absence of anamorphic stage on culture. *P. opuntiae* is of short duration, medium sized, fibrous and pure white sporocarps. Preliminary trials conducted with these two species at College of Agriculture, Vellayani indicated the scope for their large scale production (Geetha, 2015). Eventhough these mushrooms are internationally acclaimed and have excellent qualities, its production is so far not fully explored in Kerala, a place blessed with good climatic conditions and abundant agricultural wastes.

Per capita consumption of mushrooms is increasing due to increased awareness on health benefits and market expansions. Hence, more research is needed on the bioactive components in mushrooms to determine their biological responses in humans (Royse *et al.*, 2017). Mushrooms have very short shelf life and hence cannot be stored or transported for more than 24 h at the ambient conditions prevailing in most parts of year and the country. Thus appropriate postharvest practices of storage and processing are needed to avoid problems like

seasonal gluts and distress sales (Rai and Arumuganathan, 2008). Advances in research on mushroom breeding and production is very limited when compared to other crops, due to a limited knowledge on genetics and breeding systems. Sequencing of the mushroom genome and novel breeding techniques like protoplast fusion, help in combining incompatible strains of different fungal species and genera to develop somatic hybrids with higher yield and quality (Chakraborty and Sikdar, 2008). Protoplast fusion could be carried out to introduce the important traits like high biological efficiency, low temperature tolerance and shorter cropping period from either of the parents into their fusant progeny.

Hence this study "Strain improvement of oyster mushrooms- *Pleurotus cystidiosus* O.K.Mill and *Pleurotus opuntiae* (Durieu and Lev.) Sacc." was undertaken to standardize the techniques for production of oyster mushrooms, *Pleurotus cystidiosus* and *Pleurotus opuntiae*, to study their morphological, physiological and cultural characteristics as well as nutritional and organoleptic qualities and to undertake genetic improvement by protoplast fusion. Comparative studies of *P. cystidiosus* and *P. opuntiae* was also carried out with the two ruling species of Kerala *viz., P. florida* and *P. eous,* respectively.

Review of Literature

2. REVIEW OF LITERATURE

Mushroom is a macrofungus with a distinctive fruiting body which can be either epigeous or hypogeous and large enough to be seen with naked eye and picked by hand (Eliott, 1985). More than 2,000 species of mushrooms exist in nature, of which 200 species have been used as functional foods around the world, however, around 35 species have been commercially cultivated (Kalac, 2013). Mushrooms have been regarded as "gourmet cuisine" across the globe since antiquity, for their unique taste and subtle flavour. They are considered as sources of important nutrients including dietary fibre, minerals, and vitamins in particular vitamin D. Mushrooms have health-promoting benefits due to a multitude of compounds with antifungal activity, antigenotoxicity (Wang *et al.*, 2005), immunostimulation (Vaz *et al.*, 2011), hypocholesterolaemic, anti-atherogenic (Han *et al.*, 2011), stress-reducing, anti-diabetic (Akata *et al.*, 2012), antioxidative (Roupas *et al.*, 2012), antiproliferative (Zhou *et al.*, 2013), anti-tumorigenic, anti-hyperlipidemic and anti-hypertensive properties (Kim *et al.*, 2012). The commercial production of mushrooms reached about 7.96 million tonnes, in 2012, with China accounting for most of the production (5.15 million tonnes of fresh mushrooms), followed by Europe (1.87 million tonnes of fresh mushrooms) (Grujic *et al.*, 2015). Due to the increase in human population and consumption, the world demand for mushrooms is projected to grow at the rate of 15 per cent per year (Kamarudzaman *et al.*, 2015). China is leading in global mushroom production both in cultivation of edible and non-edible types with approximately 70 per cent share in world mushroom production. The second leading mushroom producing country is USA followed by European Union (Karthick and Hamsalakshmi, 2017)

2.1. GENUS PLEUROTUS

The genus *Pleurotus* (Fries) Kummer was defined by Kummer (1871). The *Pleurotus* spp. of the class *basidiomycetes* belongs to a group known as "white rot fungi" (Tsujiyama and Ueno, 2013), as they produce a white mycelium.

Pleurotus commonly known as 'oyster mushroom' are classified as follows- Phylum- Basidiomycotina; Class- Basidiomycetes; Subclass- Holobasidiomycetidae; Family- Polyporaceae; Genus- *Pleurotus*; Species- *sajor caju, sapidus, ostreatus, eous, membranaceous, florida, citrinopileatus, flabellatus, pulmonarius, geesteranus, ulmarius, tuberregium, cystidiosus, eryngii* and others. It is a cosmopolitan group of mushrooms with high nutritional value and therapeutic properties, besides a wide array of biotechnological and environmental applications (Knop *et al.*, 2015). *Pleurotus* spp. achieved third position in the production of edible mushrooms, following *Agaricus* and *Lentinula* (Fernandes *et al.*, 2015).

The genus *Pleurotus* has a worldwide distribution from temperate to tropical regions and comprises approximately 40 species found within a temperature range of 12-32 ^{0}C. Their cap is normally shell-like (about 5-20 cm in diameter; 1.9-7.8 inches) fleshy with eccentric or lateral stipe; and their color can be white, cream, yellow, pink, brownish, or dark gray found above or underground and even epiphytic (Patel *et al.*, 2012). The biodiversity of *Pleurotus* have been a main concern for numerous researchers (Zervakis *et al.*, 2012; Otieno *et al.*, 2015). Index Fungorum (http://www.indexfungorum.org) lists 202 species in the *Pleurotus* genus. *Pleurotus spp.* are known by their common names in different countries *viz.*, oyster mushroom in Europe and America, abalone mushroom in China and phoenix/dhingri mushroom in India. The other regional names are Sipi Khumbhi in Sindhi, Sadafnuma Khumbhi in Urdu, wood mushroom, dhingri, henda, kharari, shooto, meat of the forest etc.

Khare *et al.* (2010) reported various species of *Pleurotus* which are cultivated world wide *viz.*, *P. sapidus*, *P. eryngii*, *P. columbinus*, *P. cornucopiae*, *P. tuberigium*, *P. cystidiosus*, *P. fossulatus*, *P. opuntiae*, *P. citrinopileatus*, *P. membranaceus*, *P. platypus*, *P. petaloids*, *P. ostreatus* and *P. pulmonarius*. Lechner *et al.* (2011) identified three varieties in *P. djamor viz.*, var. *djamor*, var.

cyathiformis and var. *roseus*. Bazanella *et al.* (2013) stated that *P. pulmonarius* and *P. cystidiosus* are distributed in the tropical and subtropical region, while *P. eryngii* has been identified from Europe, Africa and Asia except Korea and Japan, where the commercially cultivated species is *P. ostreatus*.

BhavaniDevi (1995) carried out a macrofungal survey of Kerala, from 1985 to 1988 and made collections from 12 agroclimatic zones in four monsoon seasons. Survey revealed the presence of 134 species of mushrooms, belonging to 45 genera. Of these, *P. opuntiae, P. cornucopiae, Volvariella volvaceae, Agaricus*, *Lepiota* and *Termitomyces* spp. were the promising ones. Geetha (2011) reported *P. tuberegium* and *P. cystidiosus* from Kerala. Bhattacharya *et al.* (2012) isolated *P. ostreatus* from Wayanad district of Kerala.

Pleurotus is the well appreciated genus in Kerala, for its broad adaptability under diverse agro-climatic conditions. There are 15 to 20 species of oyster mushrooms available for cultivation in Kerala, of which the popular ones are *P. florida, P. eous, P. sajorcaju* and *P. citrinopileatus*.

2.2. *PLEUROTUS CYSTIDIOSUS*

Species which produce synnematoid fructifications (*i.e.* white synnematal columns topped with a black mucous mass of hyaline arthrospores) are taxonomically arranged in the subgenus Coremiopleurotus Hilber. The type species of this subgenus is *Pleurotus cystidiosus. P. cystidiosus* was first described by Miller (1969) on red maple tree (*Acer rubrum*) from North America. The species is known by their common names in different countries *viz.*, oyster mushroom in Europe and America, abalone mushroom/ bao yu gu in China, phoenix/dhingri mushroom in India and Ohiratake in Japan (Stamets, 1993).

Another important taxa of the subgenus Coremiopleurotus is *Pleurotus abalonus* which was first described from Taiwan. It is the first representative of *P.*

cystidiosus complex, which acquired a species-level taxonomic assignment on the basis of morphological differences (Han *et al.*, 1974). *P. abalonus* differs from *P. cystidiosus* in having thick-walled, yellow-brown cheilocystidia (thin-walled, hyaline cystidia was found in *P. cystidiosus*) and a dark-grey to dirty-brown pileus (Stalpers *et al.,* 1991). Segedin *et al.* (1995) reported that two *Pleurotus* species coming under the subgenus Coremiopleurotus *viz., P. australis* and *P. purpureoolivaceus* also produced arthro-conidia which were geographically restricted to Australia and NewZealand. Other morphologically similar species were *P. abalonus*, *P. smithii* and *P. purpureoolivaceus*. Their distinctive features include asexual synnematoid anamorphs (assigned to Andromycopsis), oblong elliptical basidiospores, clavate pileocystidia and cheilocystidia (Zervakis and Balis, 1996).

Morphological differences among members of the group, *Coremiopleurotus* are few or rather minute therefore, their taxonomic assignment has been an issue of debate. Significant ambiguities were noted concerning the exact status of *P. abalonus*, the relationships between *P. cystidiosus* and *P. smithii* (Guzman *et al.*, 1991) and the position of *P. cystidiosus* var. *formosensis* and *P. fuscosquamulosus* within the subgenus (Moncalvo, 1995). Bao *et al.* (2004) identified 5 intersterility groups in *Pleurotus viz.*, *P. ostreatus*, *P. pulmonarius*, *P. cornucopiae*, *P. cystidiosus* and *P. salmoneostramineus* complexes. *P. cystidiosus* have been cultivated for more than 40 years in Taiwan, with an estimated production of 600 MT in 2009-2010 (Peng, 2010). *P. abalonus* is a subspecies coming under the species, *P. cystidiosus*, forming a separate intersterility group (Maftoun *et al.*, 2015). Dawidowicz and Siwulski (2017) suggested that, *P. cystidiosus* is an alternative for the commercially cultivated *P. ostreatus*, as the former can be produced during the summer time, allowing its cultivation in chambers without air conditioning.

2.3. *PLEUROTUS OPUNTIAE*

Fries (1832) described and iconographed the species of *Pleurotus* found on *Opuntia maxima* as Durieu & Leville and later as *Pleurotus opuntiae*. He considered it as a synonym of the original species because of the similarities in macroscopic and microscopic characters. It was also reported from Korea, China and Japan (Singer, 1986). Ortega and Vizoso (1992) reported *P. opuntiae* from *Ricinus communis* L. and recorded it as a subspecies of *P. ostreatus*.

Hughes *et al.* (1999) reported color variants and textures in *Pleurotus djamor* and gave separate species names to such variants, *viz.*, *"P. opuntiae"* to the New Zealand grey fibrillose form, *"P. salmoneostramineus"* to the pink northeast Asian basdiocarps and *"P. ostreatoroseus"* to the rosy fruiting bodies from tropical America. Bao *et al.* (2004) demonstrated the existence of twelve discrete inter-sterility groups in *Pleurotus* through mating compatibility studies. They identified *P. opuntiae* along with *P. eugrammus*, *P. eugrammus* var. *brevisporus*, *P. sajor-caju*, *P. sapidus* and *P. florida*, as biological subspecies of *P. pulmonarius*. Shnyreva and Shnyreva (2015) revealed a partial compatibility between species within the inter-sterility group indicating the possibility for creation of new species.

Camacho *et al.* (2012) discussed the taxonomic relationships of *P. opuntiae* with other *Pleurotus* species and identified it as a close species of *P. levis*. They identified *P. opuntiae* from xerophytic temperate regions of Mexico, as parasite or saprobe on agave and opuntia. They stated *P. agaves* as a synonym of *P. opuntiae*, and *P. yuccae* as the synonym of *P. djamor*. Sub species *viz.*, *P. ostreatus* var. *columbinus*, *P. djamor* and *P. flabellatus* comes under the species *P. ostreatus*, forming the intersterility group I, whereas, *P. eugrammus*, *P. eugrammus* var. *brevisporus, P. sajor-caju, P. sapidus, P. florida* and *P. opuntiae* comes under *P. pulmonarius*, forming the intersterility group II (Maftoun *et al.*, 2015). *P. opuntiae* is highly prized in markets of Central Mexico for its good taste

and the wild strains recorded biological efficiency (BE) of more than 100 per cent (Barrales and Mata, 2016). Also they stated synonyms of *P. opuntiae viz.*, *Agaricus opuntiae*, *Dendrosarcus opuntiae*, *Panellus opuntiae* and *P. ostreatus* subsp. *opuntiae*.

2.4. SURVEY

Pleurotus species comprises of white rot fungi which are known to colonize different types of agricultural and industrial wastes, with a widespread ocurrence in various eco-geographic zones (Ragunathan and Swaminatha, 2003).

2.4.1. *P. cystidiosus*

P. cystidiosus occurs in natural areas on all continents except Antarctica and South America. However, the species is cultivated mainly in North America and Asia. Miller (1969) identified *P. cystidiosus* from red maple and later it was reported from cottonwood (*Populus deitoides*), sweetgum (*Liquidambar tyraciflua*), and asian Oaks (*Quercus nuttalli*) (Natarajan and Raman, 1984; Zervakis *et al.*, 1992). It has a marked preference to warmer climatic zones and grows on a large variety of angiosperm hosts. Moncalvo (1995) reported the occurrence of *P. cystidiosus* var. *formosensis* from Taiwan based on morphological and ribosomal DNA sequence data. Staji *et al.* (2003) reported the occurrence of new species in Asia *viz., P. cystidiosus* and *P. smithii* from *Schinus terebinthifolius* and *Morus alba* of Israel. Muruke (2014) identified *P. cystidiosus* from coastal areas of Tanzania, growing on logs of trees, producing enormous fruiting bodies, each fruiting body weighing up to one kg. Kalaw and Albinto (2014) discovered wild strains of *P. cystidiosus* from decomposing logs in Philippines during rainy season.

In India *P. cystidiosus* was first described by Natarajan and Raman (1984) from the dead logs of *Mangifera indica* of Tirunelveli, Tamilnadu. Selvakumar *et al.* (2008) collected basidiomata of *P. cystidiosus* var. *formosensis* from

decomposed wood of *Ficus bengalensis* in Chennai. Atri *et al.* (2012) described *P. cystidiosus* growing in caespitose clusters on living stems of *Mangifera indica* of Patiala, Punjab, at an altitude of 250 m. *P. cystidiosus* was recorded from dried tree logs of mango from Arippa in Western Ghats regions of Palode, Thiruvananthapuram (Vrinda, 2014) and Kollam (Geetha, 2015). Pandey *et al.* (2016) identified *P. cystidiosus* from scrub tropical forest of Hesaraghatta, Bangalore district, Karnataka at 920 m above mean sea level. Chaudhary and John (2017) reported that *P. cystidiosus* was found to be associated with bark of tulip tree (*Spathodea campanulata*) at a height of 1.5 m. Vishwakarma *et al.* (2017) identified and isolated *P. cystidiosus* from *Ficus benghalensis* of Gorakhpur.

2.4.2. *P. opuntiae*

Bresadola (1920) identified *P. opuntiae* from american agave and *Phytolacca americana*. Pegler (1977) reported *P. lueoalbus*, *P. opuntiae* and *P. flabellatus* from East Africa. Bhavani Devi and Geetha (1987) first reported the occurrence of *P. opuntiae* on oil bunch wastes from Anchal, Kerala. It is also recorded from many hosts *viz.*, *Dracaena* sp. and *Phoenix dactylifera* L. (Anastase and LaRocca, 1997).

Venturella (1991) described *P. opuntiae* as an infrequent mushroom from southernmost Italian regions, growing as parasite or saprotroph as solitary or in clusters from 0 to 500 m height on fibers of fallen cladodes of *Opuntia ficus-indica* and on trunks of *Agave americana* and *Yucca elephantipes*. Fabrizio *et al.* (2008) identified *P. opuntiae* as a saprophytic species growing on the rotting remnants of umbellifers and *Cactaceae* from spring to summer. Mondal *et al.* (2010) recorded *P. opuntiae* on dry remains of *Opuntia ficus-indica* in Aeolian Islands of Mexico during spring. Geetha (2011) recorded the occurrence of *P. opuntiae* from dried tree logs of arecanut in Western Ghats regions in Kulathupuzha, Kerala. Saha *et al.* (2012) recorded *P. opuntiae* from opuntia, agave, yucca and *Phytolacca* of Mediterranean Europe, South America, Africa

and Asia. *P. opuntiae* was seen as an occasional saprotrophe associated with *Quercus suber* and *Quercus faginea* in North Western Tunisia (Ali and Stephenson, 2016).

P. djamor also known as pink flamingo oyster or salmon or strawberry mushroom was reported from oilpalm bunch waste of Palode, Kerala (Geetha, 1993). *P. djamor* was identified from forest regions of Shimoga and Kodagu of Karnataka (Pandey and Veena, 2012), tropical moist deciduous forests of Tripura (Das *et al.*, 2014) and Western Ghat forest of Karnataka (Karun and Sridhar, 2016). Roy *et al.* (2015) identified *P. djamor* from dried *Acacia* tree logs in North Bengal.

2.5. MACROSCOPIC AND MICROSCOPIC STUDIES

2.5.1. *P. cystidiosus*

Moncalvo (1995) described the prominent characters of lignocellulosic *P. cystidiosus* var. *formosensis* with smooth edged gills, thin walled tramal cells, ellipsoid and non-amyloid spores; and unique black headed coremia. Dube (1996) described *P. cystidiosus* as a large and fleshy mushroom, which grows on tree trunks or stumps in shelf-like layers. Its pileus was shell-shaped, dark grayish brown in colour with dark brown stipe.

Atri *et al.* (2012) described the fruiting body of *P. cystidiosus* as pleurotoid, depressed, pileus of 10.5 cm diameter and 9.2 cm length, grayish brown to purplish in colour, fleshy (up to 0.3 cm thickness), with mild taste and odour. They described the lamellae of *P. cystidiosus* as yellowish white, decurrent, extending down the stipe, with wavy gill edges giving a white spore print. Its stipe is lateral, grayish brown in colour, about 4.5-6.5 cm long, 2.2-3.0 cm broad, tapering downwards with solid, flesh white underneath. Oblong, elliptical and inamyloid spores of around 8.5-14.5 µm x 4.5 -6.64 µm size, basidia of 33.2-49.8 µm x 4.98-8.30 µm size, 3.3-8.3 µm long sterigmata and

heteromorphous gill edges were recorded in *P. cystidiosus*. Clavate to pyriform, club shaped, cheilocystidia of size 16-22.5 µm x 5-8 µm, clavate to ventricose, pleurocystidia of size 36.5-43 µm x 4.98-8.3 µm and clamp connections in hyphae were abundantly found.

Vrinda (2014) described the Kerala isolate of *P. cystidiosus*. Its cap was 5-10 cm in diameter, often imbricate, pale yellow to ochraceous in colour, glabrous and characterized by squamules with undulated margin. Gills were decurrent and pale yellow in colour. Stipe was eccentric, solid, white and of size 2-4 cm × 1-2 cm. Spores were elliptic, hyaline, smooth and of size 10-15 µm × 4.5-6 µm. Pandey *et al.* (2016) described the Bangalore isolate of *P. cystidiosus*. The color of pileus was black at pinhead stage, which faded to brown at harvest stage, with an average size of 73.92 mm height and 82.60 mm width. The isolate had creamish white gills, giving a white spore print, with grayish tinge. Hyphae was monomitic, conidia was one celled and basidiospores were cylindrical of size 11-14 µm x 4-5 µm.

2.5.2. *P. opuntiae*

Wasser and Weis (1999) reported that *P. opuntiae* has medium sized pileus (20-70 mm broad) with pleurocystidia; smooth, thin, white/cream cuticle; white-cream narrowed decurrent lamellae and lateral, hard, white and felted stipe of size 1.5-3.0 cm× 1.0-1.5 cm. Basidiospores were oval and elongated of size 7.5 µm -10 µm × 3 µm -5.5 µm. *P. opuntiae* had a conchiform or flabelliforme pileus, with involuted rim in the young specimens and lobed in the mature ones. Surface of pileus was initially white in colour, with a tendency to darken towards maturity, assuming creamy whitish or gray-yellowish colorations. Pileus was slightly corrugated and tomentose decorated with small irregularly distributed scales. Its stipe was tough, fibrous and cylindrical in the eccentric-lateral position with white mycelia felt at the base (Fabrizio *et al.*, 2008).

Pandey and Veena (2012) described the isolate of *P. djamor* from Shimoga, with a pleurotoid, light pink coloured pileus of smooth margin (49.9 mm height and 33.94 mm width), stipe length of 25.4 mm and white spore print. Its hyphae was dimitic and produced cylindrical basidiospores (18.50 µm x 11.73 µm) with numerous sterile spores (22.01 µm x 12.46 µm). Sporocarps of *P. opuntiae* were initially flat-convex in diameter, then turned to depressed and funnel-shaped with slightly rolled, waved and lobed margin at maturity. Stipe was very short or nonexistent and eccentric or lateral. It had smooth, hyaline, cylindrical spores, tetrasporic basidium and cylindrical cheilocystidia (Munoz and Amaya, 2013). Sporocarp of *P. opuntiae* was convex in shape of size 2-5 cm with involuted margin and whitish, smooth cuticle. Its hymenophore was formed of fragile narrow lamellae, decurrent on the stem interspersed with lamellula. *P. opuntiae* had delicate thin, whitish meat with sweet taste and pleasant smell of fresh fruit. Basidiospores were white in colour, cylindrical to ellipsoidal, 8 µm -10 µm x 3.5 µm - 5 µm. Cheilocystidia were cylindrical in shape and 20 - 28 µm x 4 µm - 5 µm in size (Francesco, 2015).

2.6. ISOLATION AND PURE CULTURING

The pure cultures have traditionally been maintained through periodic sub-culturing and/or cold storage under refrigeration at a temperature range of 2-5 ^{0}C (Smith and Onions, 1983). Alam *et al.* (2010) stated the need for identification of pure natural strains of mushrooms for breeding, preservation of gene variability and to emphasise on their phylogenetic studies.

Asghar *et al.* (2007) revealed that joint portion was the most suitable portion of mushroom for tissue culturing as it has both the properties of stalk and veil. By cloning a piece of pure, living flesh is excised from the mushroom, placed into a sterilized, nutrient enriched medium and successful transfer results in capturing particular phenotype of the strain. Geetha (2011) collected, identified, tissue cultured and maintained the mushrooms collected from Western Ghats *viz.*,

Agaricus bitorquis, Auricularia auriculae, Calocybe gambosa, Calocybe indica, Ganoderma lucidium, Hypsizygus ulmarius, P. cystidiosus, P. eous, P. opuntiae, P. sajor-caju and P. squarrosulus using 90-95 per cent ethyl alcohol as surface sterilant. Pandey and Veena (2012) isolated and maintained pure cultures of *P. djamor* (Shimoga isolate), pink *Pleurotus* spp. (Balaghat isolate), white *Pleurotus* spp. (Balaghat and Madurai isolates) and *P. cystidiosus* (Bangalore isolate), using 0.1 per cent mercuric chloride as the surface sterilant. Sardar *et al.* (2016) pure cultured six different *Pleurotus* strains collected from the Punjab province *viz.*, *P. ostreatus*, *P. sajor-caju*, *P. eryngii*, *P. columbinus*, *P. sapidus* and *P. florida*, using 5 per cent sodium hypochlorite as sterilant on malt extract agar (MEA) slants.

2.7. MOLECULAR CHARACTERIZATION

Molecular techniques especially ITS (Internal Transcribed Spacer) sequencing is widely used for identification, characterization and phylogenetic studies of mushrooms (Fonseca *et al.*, 2008). The adoption of biochemical and molecular approaches have brought some clarifications for species delimitation in the *Pleurotus* genus when combined with morphology and sexual compatibility studies (Menolli *et al.*, 2014).

Zervakis *et al.* (2004) used ITS phylogeny and mating data to distinguish between *P. australis* and *P. cystidiosus*. Within *P. cystidiosus,* ITS phylogeny showed a deep split between Old and New World isolates. They reported that, Asia-Pacific strains of *P. abalonus, P. cystidiosus* and *P. cystidiosus* var. *formosensis* were not phylogenetically distinct from each other. However, they formed a phylogenetically distinct group from Coremiopleurotus. Li *et al.* (2007) found that 28S rDNA sequencing distinguished monomitic species *viz., P. tuberregium* and *P. levis* from other species of *Pleurotus, viz., P. djamor, P. calyptratus* and *P. opuntiae*. Chandra *et al.* (2010) studied the genetic divergence of eight Indian species of *Pleurotus viz. P. florida*, *P. membranaeceus*, *P. sajor-caju*, *P. djamor*, *P. cystidiosus*, *P. flabelletus*, *P. sapidus* and *P. ostreatus* based

on ITS and random amplified polymorphic DNA pattern. Dung *et al.* (2012) identified white and Japanese oyster mushrooms from Vietnam as *P. floridanus* and *P. cystidiosus* respectively using ITS primers.

Avin *et al.* (2012) identified *P. pulmonarius*, *P. citrinopileatus*, *P. floridanus*, *P. sapidus*, *P. cystidiosus* and *P. eryngii* through ITS sequencing and accession numbers were retrieved from GenBank. Menolli *et al.* (2014) used ITS sequencing to confirm the morphological identity of *P. albidus, P. djamor, P. fuscosquamulosus, P. pulmonarius* and *P. rickii*. Hussain *et al.* (2015) sequenced ITS regions (ITS1-5.8S-ITS2) of *P. cystidiosus* collected from subtropical pine forests of Pakistan and taxon was identified as a new species of Pakistan.

2.8. PHYSIOLOGICAL STUDIES

Physiological studies are the studies of responses of the organisms to their environment, the mechanisms involved in their growth, development, reproduction and nutrient uptake. Knowledge of physiology is of great importance to mushroom growers, as careful daily management of mushroom crop depends upon the knowledge of the physiological needs of the fungus during different stages of development (Chang and Miles, 2004).

2.8.1. Growth in different medium

Stamets (1993) suggested mannitol egg yolk polymyxin agar, corn meal yeast glucose agar, dog food agar and potato dextrose yeast agar as suitable culture media for the optimum growth of *P. cystidiosus* mycelium. Mycelial growth of *P. ostreatus*, *P. sajor-caju*, *P. cystidiosus* and *V. volvacea* were maximum in MEA, followed by Murashige and Skoog's medium and PDA (Nasim *et al.*, 2001). Maximum mycelial growth of *P. sajor-caju* was obtained on MEA compared to PDA, at 25^{0}C (Asghar *et al.*, 2007). Selvakumar *et al.* (2008) reported that growth rate of Chennai isolate of *P. cystidiosus* on PDA was 0.45 mm day^{-1}. Lechner *et al.* (2011) studied the mycelial growth of *Pleurotus* spp. on Noble's medium at 25^{0}C. *P. pulmonarius* showed the fastest growth (7.4 mm day^{-}

$^{-1}$), followed by *P. albidus* (6.16 mm day^{-1}), *P. ostreatus* (5.8 mm day^{-1}), *P. cystidiosus* (1.2 mm day^{-1}) and *P. djamor* (0.5 mm day^{-1}).

Rawte and Diwan (2011) recorded maximum biomass production of *P. florida, P. sajor-caju, P. eous and P. flabellatus* on PDA followed by MEA, Richard's Broth, Czapecks' Dox and Asthana Hawker's media. *P. cystidiosus* showed a cottony and scattered mycelium growth on PDA (Atri *et al.*, 2012). Isolate of white *Pleurotus* spp. from Balaghat produced maxium mycelium (dry weight basis) in wheat extract agar (4.91 mg), followed by Roderquez Caceras medium (4.61 mg), rose bengal agar (4.39 mg), MEA (3.94 mg) and PDA (3.74 mg) (Pandey and Veena, 2012). Hoa and Wang (2015) identified that PDA, yeast dextrose agar, sweet potato dextrose agar and MEA were equally good in supporting the mycelium growth of *P. cystidiosus*. Pandey *et al.* (2016) identified wheat extract agar followed by rice bran agar and MEA as the best media for *P. cystidiosus*, giving mycelial dry weights of 4.91, 4.39 and 3.94 mg respectively. Dawidowicz and Siwulski (2017) identified maltose agar medium, followed by synthetic Hansen's medium as the best media for the growth of *P. cystidiosus* showing a mutual dependence between morphological and qualitative characteristics, which enabled production optimization.

2.8.2. Growth at different temperature

Temperature has a profound effect both on growth and reproduction of fungi (Kaul *et al.*, 1978). The genus, *Pleurotus,* because of its flexible temperature and other environmental requirements has more cultivated species than any other mushroom (Zadrazil and Dube, 1992).

The mycelial growth variation of six species of *Pleurotus* propagated on PDA medium at different temperatures was studied by Mehta and Bhandal (1988). They found, maximum colonization within a temperature range of 15-30 ^{0}C, whereas, no growth was observed at 10, 35 and 40 ^{0}C. Cangy and Peerally (1995) reported that 10 different species of *Pleurotus* exhibited maximum growth rate

(14-15 mm day^{-1}) at 25-30 ^{0}C. Fan *et al.* (2000) recorded mycelial growth at the rate of 9.68 mm day^{-1} and 43.4 mg $plate^{-1}$ in 9 days at 24 ^{0}C for *P. ostreatus*. The optimal growing temperature, pH and C/N ratio of *P. cystidiosus* were 25 to 30 ^{0}C, 7-9 and 20:1 respectively (Jang *et al.*, 2003). Zharare *et al.* (2010) found that *P. sajor-caju* could tolerate a high temperature of 35 ^{0}C. *P. djamor* (Shimoga isolate) showed maximum mycelial growth at 25 ^{0}C (12.85 mm day^{-1}) followed by 30 ^{0}C (11.07 mm day^{-1}), whereas white *Pleurotus* spp. (Balaghat isolate) showed mycelial growth rates of 15.0 and 14.91 mm day^{-1}, respectively (Pandey and Veena, 2012). Hoa and Wang (2015) reported that *P. cystidiosus* showed maximum colony diameter at a temperature of 28 ^{0}C. *P. ostreatus*, *P. sajor-caju*, *P. eryngii*, *P. columbinus* and *P. sapidus* showed maximum growth rate at 25 ^{0}C while *P. sapidus* showed maximum growth at 35 ^{0}C (Sardar *et al.*, 2016).

2.8.3. Growth in different pH

Every mushroom has its optimum pH range and it varies with its stage of development. Mycelial growth is favoured by a pH range between 4 and 7, whereas, pH range of 3.5 to 5 favours the formation of basidiocarp (Urben, 2004).

Pleurotus spp. *viz. P. sajor-caju*, *P. flabellatus* and *P. ostreatus* grew well at pH 5.5 (Suhorban and Nair, 1994). Singh and Kushwaha (2007) identified pH 7 to be the most appropriate for growth of *H. ulmarius*. Maximum biomass of *P. florida*, *P. sajor-caju, P. eous, P. flabellatus, P. sp.* was recorded at pH 5 when cultured on PDA (Rawte and Diwan, 2011). *P. djamor* (Shimoga isolate) produced maximum mycelium on dry weight basis at pH 6.5 (275.43 mg) followed by 7.5 (272.2 mg) and 8 (250.63 mg). White *Pleurotus* spp. (Balaghat isolate) recorded maximum growth at pH at 8 (289 mg), followed by 7 (258.2 mg) and 5.5 (229.8 mg) (Pandey and Veena, 2012). Szarvas *et al.* (2014) reported that, 13 wild *P. eryngii* strains of Hungarian origin grew faster on two optimum pH values *viz.*, the acidic pH 4.5 and alkaline pH range of 7.5 to 8.5. Sardar *et al.* (2016) noted that, *P. ostreatus*, *P. sajor-caju*, *P. eryngii*, *P. columbinus* and *P.*

sapidus exhibited maximum mycelial growth at pH 6 whereas minimum mycelial growth was recorded at pH 4 at 25 ^{0}C.

2.8.4. Growth in light and dark conditions

Zandrazil (1982) reported that absence of light facilitated mycelial growth in oyster mushrooms while alteration of light and darkness resulted in increased number of fruiting bodies. Sharma (2004) studied the effect of light period on mycelial growth of *P. djamor* and found the highest growth in complete darkness. Truong *et al.* (2006) observed high amount of coremium production in the subgenus Coremiopleurotus under 400 Lux light intensity at 23 ± 1 ^{0}C. The study revealed that the pigmentation was induced by light. Selvakumar *et al.* (2008) incubated agar plate at continuous light illumination for high amount of pigment (coremia) production by *P. cystidiosus*. Maximum radial growth of *P. djamor* (75.33mm) was recorded under 6 h light and 18 h dark period whereas, minimum radial growth was observed under 24h light (Chauhan, 2014). *P. citrinopileatus* (89.07 mm) and *P. djamor* (88.86 mm) incubated in lighted condition exhibited larger mycelial diameter while *P. salmoneostramineus* (89.26 mm) exposed in dark condition produced larger mycelial diameter (Jacob *et al.*, 2015). *P. eous*, *P. florida*, *P. sajor-caju*, *P. citronopileatus*, *P. fossulatus*, *P. flabellatus, P. platypus*, *P. ostreatus*, *P. ulmarius* and *P. sajor-caju* exhibited highest linear growth at 200 lux light intensity. Their growth declined substantially as the light intensity increased from 200 to 1000 lux, whereas growth was found to be moderate in darkness. *P. citrinopileatus* and *P. eous* showed the highest (78.89 mm) and lowest (63.92 mm) mycelial growth at 200 lux light intensity (Rout *et al.*, 2015). Sumi (2016) revealed that *H. ulmarius* took minimum time for complete mycelial growth under dark condition (8.9 days) compared to light conditions (8.4 days).

2.8.5. Role of amendments on mycelial growth

Stamets (1993) recorded maximum mycelium growth of *Pleurotus* spp., when culture medium was amended with 1 per cent organic acid and 0.5 per cent yeast extract. Supplementation of MEA, PDA, corn meal agar (CMA), glucose

agar (GA) and oat meal agar (OMA) with 0.1 per cent peptone stimulated the rate of mycelial growth and improved the quality of the projected mycelial mat of *Pleurotus* spp. He also suggested peptone, oatmeal, oat bran, rye or wheat flour, soybean meal, spirolina and high quality dry dog food as suitable nitrogen/carbohydrate supplements. Fasidi and Olorunmaiye (1994) identified that yeast extract significantly improved the mycelial growth of *P. tuberregium* followed by asparagine, casein, glycine and calcium nitrate. However, sodium nitrate, potassium nitrate and ammonium sulphate inhibited the mycelial growth. Similarly, thiamine, pyridoxine, gibberellic acid (GA) (1 and 10 ppm), 2,4-dichlorophenoxy acetic acid (2,4-D) (10 ppm), Ca, K, Cu, and Zn relatively supported the mycelial growth.

Eswaran and Ramabadran (2000) conducted physiological studies on *P. eous* and recorded its best radial growth on PDA, whereas, the highest mycelial weight was recorded in media amended with glucose and asparagine. They identified thiamine (50 ppm), GA (5 ppm) and copper sulphate (3 ppm) as the best vitamin, growth regulator and trace element respectively for the growth of *P. eous*. Chang and Miles (2004) revealed that trace elements *viz*., calcium, zinc, manganese, iron, copper and molybdenum could supplement the culture media of mushrooms. Adenipekun and Gbolagade (2006) reported that thiamine was the most suitable vitamin for the mycelial growth of *P. florida* (146.7 mg/30cm^3) followed by pyridoxine (133.3 mg/30cm^3), cobalamine (100 mg/30 cm^3) and folic acid (86.7 mg/30cm^3). They also identified 0.1 per cent GA (130 mg/30cm^3) as the best phytohormone followed by 0.1 per cent indole acetic acid (IAA) (101.7 mg/30 cm^3) and 1 per cent 2,4-D (100 mg/30 cm^3). Calcium and zinc were identified as the best macro and micronutrient for its mycelial growth.

Hypsizygus sp. and *A. bisporus* exhibited maximum mycelial growth rates of 1.049 and 0.306 cm day^{-1} respectively when grown on complete yeast media (Rashid *et al*., 2010). Maximum radial growth of *P. sajor caju* (8.56; 9.00

cm), *P. florida* (9; 8.75 cm), *P. flabellatus* (8.26; 8.50 cm), *P. fossulatus* (8.45 and 8.93 cm) and *P. sapidus* (9.00 and 8.50 cm) was observed on PDA supplemented with ferrous sulphate and copper sulphate 1 per cent each respectively (Kumar *et al.*, 2011). Chauhan (2014) identified potassium nitrate, 50 ppm kinetin and maltose as the best nitrogen source, growth regulator and carbon source respectively which supported maximum mycelial growth of *P. djamor*. *P. ostreatus* exhibited optimum mycelial growth and biomass production when cultured on a special medium containing iron at a concentration of 150 mg L^{-} (Almeida *et al.*, 2015). Hoa and Wang (2015) revealed that, glucose, dextrose, and sucrose were the carbon sources which gave good mycelium growth of *P. cystidiosus*. Also, amendment of PDA, with 0.03-0.05 per cent ammonium chloride increased the colony diameter of mushroom mycelium. Ogidi *et al.* (2016) revealed that mycelial growth rate and biomass production of *Pleurotus* species significantly decreased as the iron concentration increased from 0 to 100 mg L^{-1}.

2.9. SUBMERGED CULTURING OF *P. CYSTIDIOSUS* AND *P. OPUNTIAE*

Lindequist *et al.* (2005) revealed that 80 to 85 per cent of all edible medicinal mushroom products were derived from the fruiting bodies and only 15 per cent were initiated from mycelia extracts. However, production of fruiting bodies is effortful and time consuming as it demands large volumes of substrate, space and skilled labour. On the other hand, cultivations which are performed in vegetative phase are more reliable as they can be done on a small and medium scale with controlled parameters such as temperature, humidity, pH and aeration (Inacio *et al.,* 2015). Thus, submerged cultivation is a promising and under explored alternative for the extraction of bioactive molecules in short time.

Submerged cultivation also allows mycelia storage for a long period without genetic alterations, enabling the conservation of biodiversity (Zilly *et al.*, 2011). Elisashvili (2012) reported that fruiting bodies and culture broth of

mushrooms contain secondary metabolites including polysaccharides, proteins, phenolic compounds, polyketides, triterpenoids, steroids, alkaloids and nucleotides. Arango and Nieto (2013) carried out submerged liquid fermentation with *Pleurotus* sp. and identified potato dextrose broth (PDB) (pH 4-6), amino acids, reducing sugars (glucose and xylose), casein hydrolyzate, soybean cake, yeast extract and peptone as the main carbon and nitrogen sources. They standardised the culture conditions at a temperature of 25-30°C with an agitation ranging from 100 to 160 rpm. Soares *et al.* (2013) reported that prior to using mycelial biomasses, it is necessary to prove that they are similar to fruiting bodies. Chauhan (2014) recorded maximum mycelial weight of *P. djamor* in Glucose-asparagine solution (805.7 mg). Dulay *et al.* (2015) standardised the liquid culture conditions (culture broth, pH, temperature and shaking condition) of *P. cystidiosus* for mycelial biomass production and reported SDB with pH 6 as the most suitable culture medium for maximum mycelial biomass when incubated at 28 ^{0}C.

2.10. COREMIAL CHARACTERS OF *P. CYSTIDIOSUS*

Many mushrooms have alternative asexual life cycles. The advantage of asexual reproduction is that, it is not as biologically taxing as mushroom formation. Asexual reproduction disperses spores under a broader range of conditions than the rather stringent parameters required for mushroom formation. In essence, asexual reproduction represent short-cuts in the mushroom life cycle. Asexual spores are produced much in the same manner as mold spores-on microscopic tree-like structures called conidiophores. Spores can also be found embedded within the mycelial network. Oidia, chiamydospores and coremia are some examples of asexual reproduction. In culture, these forms appear as "contaminants" confusing many cultivators. An excellent example, in this category is the abalone mushroom, *P. cystidiosus* and allies.

Miller (1969) found that, *P. cystidiosus* has black headed coremoid imperfect stage seen on the culture and edge/face of lamella. Its close relatives *P.*

abalonus and *P smithii* also produce white mycelia which become speckled with black droplets (Stamets, 1993). Capelari (1999) assigned anamorphic state of *P. cystidiosus* to a hyphomycete *viz.*, *Antromycopsis macrocarpa*. *P. cystidiosus* produced imperfect coremoid stage, composed of dark mucous mass of hyaline, dikaryotic arthrospores on the mycelium with the remnant of a clamp connection. Continuous cell extension and division in the coremium stipe supplied the cells for arthroconidiation at the coremium apex which is surrounded by a coremioliquid droplet (Selvakumar *et al.*, 2008). Atri *et al.* (2012) described *P. cystidiosus* subsp. *abalonus* as a wood decaying edible basidiomycetous mushroom characterized by the formation of specialized anamorphic structures called coremia. The aerial mycelium of the mushroom (anamorph) form toxocyst which bears coremial spores of size 7.77 µm- 10.81 µm. *P. cystidiosus*, following inoculation on PDA medium, irregular mycelia growth started on the entire inoculated tissues after four days. Initially, a large amount of hyaline, aerial mycelia appeared which in due course became whitish. Coremia formation started in the form of small swellings on the entire tissues and tiny watery droplets having blackish colour appeared on the white stalk which terminated into distinct capitate structures called toxocysts. Mycelial growth was irregular and cottony and after a series of subculturing, mycelia was freed from the toxocysts (Juma *et al.*, 2015).

2.10.1. Isolation of melanin

Melanins are macromolecules formed by oxidative polymerization of phenolic or indolic compounds. Based on colour and structural classes, there are three types of melanins *viz.*, eumelanins, pheomelanins and allomelanins. Eumelanins are black to brown coloured pigments produced by melanisation through classic Mason-Raper pathway (Tarangini and Mishra, 2013). Melanin pigment from natural sources like microorganisms is an attractive choice for commercial scale production.

Melanin pigments are composed of different types of monomer units which are connected through strong carbon–carbon bonds (Prota, 1992) which makes its systemic characterization very difficult. Casadevall *et al.* (2000) characterised melanins by their relative resistance to degradation by hot acids, hot concentrated alkaline solutions and bleaching by strong oxidizing agents. Selvakumar *et al.* (2008) isolated and characterized melanin pigment from *P. cystidiosus* var. *formosensis* through bio-chemical analysis. Vallimayil and Eyini (2013) extracted the extracellular melanin from *P. djamor* and *P. citrinopileatus*. The melanin gave peaks at UV (460 and 490 nm) and IR region (3424.96 and 3411.46 cm^{-1}). Absorbance spectrum of the extracellular melanin extracted from *Schizophyllum commune* showed characteristic peaks in the UV (325 and 341 nm) and IR region (3431.8 cm^{-1}) (Arun *et al.*, 2015).

2.10.2 Antioxidant activity of melanin

Melanin particle possess antioxidant property in biological systems. It can scavenge free radicals and has the ability to sequester redox active metal ions (Ju *et al.,* 2011). Antioxidant activity is analyzed by 1,1-diphenyl-2picryhydrazyl (DPPH) free radical scavenging assay. It relies on the reduction of methanolic DPPH solution in the presence of a hydrogen donating compound (antioxidant). This method is widely used as it is the simplest and most accurate method to evaluate the radical scavenging ability of antioxidants (Abdulla *et al.*, 2013).

Melanin complex from medicinal mushrooms *viz.*, *Inonotus obliquus* (Chaga) (Babitskaya *et al.*, 2002) and *Auricularia auricila-judae* (Revskaya *et al.*, 2012) showed high antioxidant and free radical scavenging activities. Arun *et al.* (2015) revealed that when concentration of extracellular melanin produced from *S. commune* was increased from 10 to 50 µg, the DPPH scavenging activity also increased from 87 to 96 per cent. Fractions I and II from the melanin of *A. auriculae* fruiting body (AAFB) exhibited strong antioxidant activities with IC 50 values of 0.20 and 0.17 mg ml^{-1} respectively (Zou *et al.*, 2015).

2.11. SPAWN PRODUCTION

Mushroom spawns are similar to seed of plants and the term spawn is derived from old French verb "*espandre*" meaning to expand. Spawn is a substrate in to which mushroom mycelium has been impregnated and developed to serve as the main inoculum or seed for the substrate in mushroom cultivation (Chang and Miles, 2004). Failure to achieve a satisfactory harvest may often be traced to unsatisfactory spawn used (Chang, 2008). Cultivation process of oyster mushrooms include three main steps *viz.*, isolation of mushroom fungus from fruiting bodies, preparation of primary and secondary spawn followed by cultivation using secondary spawns for production of fruiting bodies (Dung *et al.*, 2012).

The first pure culture spawn was produced for *A. bisporus* in France on horse manure compost (Constantin and Matruchot, 1894). The process of making spawn on grain was first introduced by Sinden (1934) and suggested grains such as sorghum, millet, rye, paddy or wheat which are rich in protein and carbohydrate. Sawdust spawn is mainly used to inoculate a "fruiting substrate", typically logs or sawdust supplemented formulas (Stamets, 1993). Pathmashini *et al.* (2008) recorded BE of 25.38, 30.76, 16.57 and 11.99 per cent for *P. albidus*, *P. ostreatus* and *P. pulmonarius* when sorghum, pearl millet, maize and paddy grains were used as spawn substrate; and took 8 to 12, 8 to 10 and 5 to 10 days respectively for spawn production. *P. opuntiae* took 10.20 and 15.20 days for spawn and mother spawn production respectively whereas *P. florida* took 15.80 and 20.60 days respectively, when paddy grain was used as substrate. *P. eous* required 12 and 15.40 days for spawn and mother spawn production respectively when paddy grain was used as substrate (Geetha, 2011). Mycelial spread of *P. djamor* and *P. cystidiosus* took 11 to 12 and 15 to 20 days respectively to completely colonize the wheat grains (Lechner *et al.*, 2011). Tinoco *et al.* (2011) found that larger surface area and pore of substrates provided better supports for the mycelial growth.

Narh *et al.* (2011) found that, a combination of sorghum and millet at 3:1 ratio was the best substrate for spawn production of *P. ostreatus*. Many workers have used sawdust from different sources for the preparation of mushroom spawns *viz.*, rubber wood sawdust for *P. eryngii* and mixed wood sawdust for *P. (flabellatus) djamor* (Khan *et al.*, 2013), sawdust from *Albizia saman* (rain tree) for *P. ostreatus*, (Bhattacharya *et al.*, 2014), mango sawdust for *P. cystidiosus* (Hoa *et al.*, 2015) and oak sawdust for *Ganoderma lucidum* (Joshi, and Sagar, 2016). Hoa and Wang (2015) identified brown rice as the most favorable grain for the mycelial growth of oyster mushrooms followed by yellow corn and wheat.

2.11.1 Role of amendments on spawn growth

Stoller (1940) proposed that flocculating agents *viz.*, finely ground or powdered gypsum, superphosphate (a mixture of gypsum and calcium phosphate), calcium chloride, diatomaceous earth, iron, aluminium, manganese and magnesium sulphate can be added to the spawn substrates which permit the granular mixture to serve as a substrate without forming a sticky paste or cement-like product, thus enabling faster mycelial growth. Also salts of aluminium, iron, manganese, chromium and zinc can be added to the spawn substrate which will increase the hydrogen ion concentration of the substrate without hindering the mycelial run. Impens (1973) recommended the use of thiamine as an absolute requirement for the spawn run of *V. volvaceae*.

Ascorbic acid, folic acid and riboflavin did not support the mycelial growth of *P. tuberregium* (Fasidi and Olorunmaiye, 1994). Supplementation of sawdust spawn with rice bran 4 per cent and calcinated oyster shell powder 2 per cent increased the calcium content in the fruiting body of *P. eryngii* (315.7 mg 100 g^{-1}), without extension of time taken for spawn run and primordial formation (Choi *et al.*, 2014). Gupta and Sharma (2014) prepared spawns of *P. sajor caju*, by impregnating paddy grains with 10 per cent calcium carbonate ($CaCO_3$) and 10

per cent calcium sulphate ($CaSO_4$) on dry weight basis. Hoa *et al.* (2015) prepared spawns of *P. cystidiosus* and *P. ostreatus*, using acacia sawdust, supplemented with rice bran 9 per cent, sugar 1 per cent, $CaCO_3$ 1 per cent, ammonium chloride 0.03 per cent, magnesium sulfate 0.03 per cent and mono-potassium phosphate 0.03 per cent.

Kumar (2015) found that, application of tetracycline (50 µg/kg) during spawn preparation, reduced the bacterial contamination up to 98.33 per cent. Thongklang *et al.* (2015) identified, maximum mycelium growth of *P. ostreatus* in spawns prepared from sorghum mixed with corn cobs (16.83 mm/day), followed by sorghum mixed with rice husks (11 mm/day) and sorghum alone (8.11 mm/day). Various other spawn supplements have been proposed by many workers *viz.*, 1.5 to 2 per cent $CaSO_4$ (Vetayasuporn, 2016) and 2 per cent $CaCO_3$ (Khatoon and Sharma, 2017). *P. djamor* gave the maximum yield when spawns amended with glucose (613.33g/kg of dry substrate) was used, followed by sucrose (500 g/kg) (Satpal *et al.*, 2017).

2.12. CULTIVATION

Most of the basidiomycetous fungi have two distinct phases in their life cycle: the vegetative phase represented by the mycelium and the reproductive phase represented by fruiting bodies, the basidiocarps (Zadrazil, 1978). Bioconversion of lignocellulosic un-composted agro-wastes through cultivation of *Pleurotus* spp., results in the utilization of renewable resources for the production of protein rich fruiting bodies which in turn can sustain the food security of people (Naraian *et al.*, 2010).

Zadrazil (1978) detailed the cultivation of *P. ostreatus* on wooden stumps. Substrates used in mushrooms cultivation have direct effect on chemical, functional and sensorial characteristics of mushrooms produced (Oyetayo and

Ariyo, 2013). Alemu and Fisseha (2015) and Siqueira *et al.* (2016) have identified many suitable substrates for the cultivation of oyster mushrooms including paddy straw, maize stalks/cobs, vegetable plant residues and bagasse.

Miller (1969) induced *P. cystidiosus* to fruit on an inoculated wooden block maintained under humid conditions. Jong and Peng (1975) first reported the commercial cultivation of *P. cystidiosus* in Taiwan. Quimio (1986) standardized the cultivation of *P. cystidiosus* on paddy straw and sawdust in Philippines. Stamets (1993) identified that strains of *P. cystidiosus* from Thailand and Taiwan produced higher crops on rice straw and lower yields on wheat straw. Lechner *et al.* (2011) reported poor yields of *P. cystidiosus* with BE values of 0 and 30 per cent when wheat straw and sawdust from *Salix* sp., respectively was used as bed substrates. Kalaw and Albinto (2014) produced fruiting bodies of *P. cystidiosus* when they used a substrate consisting of 7 parts rice straw and 3 parts saw dusts. Hoa *et al.* (2015) reported that corn cob and sugarcane bagasse were the most suitable substrates for cultivation of *P. cystidiosus* which gave the highest values for cap diameter, stipe thickness, mushroom weight, yield, biological efficiency, protein, fiber, ash and mineral content (Ca, K, and Mg). Substrate formulas *viz.*, 100 per cent corn cob, 100 per cent sugarcane baggase, 50 per cent corn cob + 50 per cent sawdust and 50 per cent sugarcane baggase + 50 per cent sawdust, resulted in the maximum mycelium growth and biological efficiency (36.2 to 50.14 per cent) of *P. cystidiosus* (Hoa and Wang, 2015).

Bahukhandi and Munjal (1989) detailed the cultivation of wild species of *P. opuntiae* growing on the decaying stumps of *Opuntia* species from Garhwal (Himalayas). Geetha (2015) recorded an average yield of 590 g kg^{-1} of paddy straw for *P. opuntiae* in a shorter period of 13.60 days. She found that *P. florida* took 18.6 days for mushroom production and gave a total yield of 920.66 g kg^{-1} of paddy straw. Barrales and Mata (2016) recorded the highest BE of *P. opuntiae* in 1:1 barley straw: sugarcane leafs (105.21 per cent) and lowest in barley straw (4.43 per cent). Shoba and Krishnakumari (2017) found that *P. eous* recorded a

BE of 100-110 per cent when paddy straw was used as bed substrate. Sumi (2016) revealed that *H. ulmarius* showed minimum BE in neopeat (31.5 per cent).

2.12.1. Role of amendments on mushroom production

The agricultural wastes commonly used are very poor in nitrogen content therefore it is a common practice to supplement the substrates with different nitrogen rich compounds for increased mushroom yield and nitrogen content in the sporophores. The deficiency of several nutrients is improved by the deliberative supplementation of external compounds (Naraian *et al.*, 2010).

The commonly used supplements are sources of organic nitrogen mainly cereal bran, whose quantity and kind varies according to the species or strain of mushroom and the growth stage (Rajarathnam *et al.*, 1986). The cereal bran supplementation promoted the secretion of cellulase, hemicellulose and laccases which resulted in the degradation of cellulose, hemicellulose and lignin respectively. The degraded carbohydrates served as energy sources for constructing the structural components of the fruiting body (Peng *et al.*, 2000).
It was reported that many amendments *viz.*, wheat bran, cotton meal, brewer's grain, wood dust, chicken manure, yeast mud, cotton seed cake, urea (Naraian *et al.,* 2010), wheat bran (Moonmoon *et al.*, 2010) rice bran (Chae and Ahn, 2013) and soybean flour (Jeznabadi *et al.,* 2016) gave better yield of oyster mushrooms.

P. cystidiosus could be cultivated in southern yellow pine chips with a BE of 54 per cent and supplementation with 20 per cent wheat grain increased the BE to 100 per cent (Croan, 2003). Jang *et al.* (2003) reported that pine sawdust substrate supplemented with 20 per cent rice bran and calcium carbonate recorded a higher yield for *P. cystidiosus* in the bottle method of cultivation. Narayanasamy *et al.* (2010) recorded a higher yield of *P. florida*, when cotton waste mixed with wheat bran was used as substrate, compared to paddy straw at 23^0C and 80 per cent humidity. Generation of fruiting bodies of *P. djamor* was significantly

augmented, when cladodes after hosting cochineal were added to the growing substrate (Portillo, 2013). Amendment of paddy straw with neemcake, oil seed cake, wheat bran and cow dung resulted in higher BEs of 74.33, 71.56, 64.21 and 12.32 per cent respectively for *P. ostreatus* (Tesfaw *et al.*, 2015). Thongklang *et al.* (2015) identified sawdust + rice husks as the optimal substrate for the cultivation of *P. ostreatus* which resulted in 277.50 g of fruiting bodies in a 40 day production cycle. Kwagyan *et al.* (2016) observed that supplementation of paddy straw with rice bran could not enhance the mushroom yield of *P. eous* and the use of 4-8 days composted substrate recorded the highest BE of 67.1-75.1 per cent. Supplementation of the maize stalk residues with 14 per cent maize flour and 12 per cent wheat bran increased the BE of *P. ostreatus* (Mkhize *et al.*, 2016).

Supplementation of rice straw with 2 per cent urea completely inhibited the mycelial growth of mushrooms due to change in pH by the release of ammonia (Kausar and Iqbal, 1994). Spraying of sugarcane bagasse with 0.1 per cent urea increased the yield of *P. ostreatus* however, yield decreased as the concentration of urea increased to 0.5 per cent (Kanhar *et al.*, 2007). Deo and Kanani (2015) identified that *P. ostreatus* gave the maximum yield, when maize cobs supplemented with 100 g of urea (1993 g $tube^{-1}$ of spawn) was used as bed substrate, followed by maize cobs with 75 g of urea (1833 g $tube^{-1}$ of spawn).

Corn cobs combined with 20 per cent wheat bran, 20 per cent buck wheat hull, one per cent glucose and one per cent $CaSO_4$, gave the highest BE for *P. cornucopiae* (Guo-meilan *et al.*, 2010). Thongsook and Kongbangkerd (2011) reported that spraying of substrate with 5 per cent $CaSO_4$ resulted in large-sized caps of *P. ostreatus* whereas $CaSO_4$ at 10 per cent decreased the total yield of mushroom. Spraying of mushroom beds with fertilizer mixture composed of 1.15 g urea, 4.38 g potassium di-hydrogen phosphate and 94.47 g cellulose powder recorded the hig hest yield in *P. florida* (780 g kg^{-1} substrate) and *P. sajor-caju* (828 g kg^{-1} substra te) followed by the supplements *viz.*, wheat bran and rice bran (Patil *et al.*, 2014).

Purkayastha and Chandra (1974) reported that application of 0.01 ppm IAA and 0.01 ppm GA significantly improved the mycelial growth of *Calocybe indica* whereas 100 ppm IAA and 10 ppm GA gave the highest BE. Tomita *et al.* (1984) revealed that GA at 1 mg L^{-1} increased the biomass and protein content of oyster mushrooms by 16 and 2.8 per cent respectively whereas GA at 3 mg L^{-1} decreased the growth and protein content of mushrooms. Rajarathnam *et al.* (1986) reported that ferrous sulphate significantly augmented the yield of *P. flabellatus* when sprayed to the paddy straw after spawn run. Neelam *et al.* (2013) reported that ammonium chloride improved the spawn run of *P. florida* and *P. ostreatus* compared to sodium nitrate and calcium nitrate because nitrate ions have inhibitory effect on fungal membranes. Spraying of wheat straw beds of *P. opuntiae* with 1 per cent coelomic fluid and 5 per cent nutriwash increased the yield by 11.7 and 18.4 per cent respectively (Sharma and Deeba, 2014). Hoa and Wang (2015) identified an increased mycelium growth in *P. cystidiosus* and *P. ostreatus* with increased concentration of ammonium chloride.

2.13. CROPPING CONDITIONS

Cropping conditions for fruiting phase are quite different from that of spawn run phase. The key factors for primordia formation and development of sporophores in the cultivation of *Pleurotus* are concentrated to the following ecological factors: (1) temperature, (2) relative humidity (RH) (3) aeration (carbon dioxide (CO_2) and oxygen (O_2) concentration) (4) light.

Quimio (1986) identified that *P. cystidiosus* required a fruiting temperature of 25-28^{0}C. A temperature of 18-24 ^{0}C, 95-100 per cent RH, 500-1000 ppm of CO_2 and 1000-2000 lux of light per hour of fresh air exchange is required for the primordia formation of *P. cystidiosus*. An incubation temperature of 24-30^{0}C, 90-95 per cent RH, 5000-20,000 ppm of CO_2 and absence of light is needed for the spawn run of *P. cystidiosus*. A temperature of 21-27^{0}C, 85-90 per cent RH, <2000ppm CO_2 and 500-1000 lux of light are required for its fruiting

body production (Singer, 1986). A temperature of 21-27^{0}C, 85-90 per cent RH, <2000 ppm of CO_2, 500-1000 lux of light and 4-5 per hour of fresh air exchange is required for the development of fruiting body of *P. cystidiosus* (Stamets, 1993). Hoa and Wang (2015) reported that, *P. djamor* required incubation temperature, induction temperature, frutification temperature and harvest temperature of 15–35, 18–25, 24–30 and 20–30^{0}C, respectively, whereas, *P. abalonus* required 15–35, 12–18, 20–30 and 25–30^{0}C, and *P. cystidiosus* recorded temperatures of 18-33, 18-24, 21-28 and 22-28 ^{0}C respectively. Shnyreva *et al.* (2017) found that, *P. cystidiosus* required temperatures of 18±1 ^{0}C and 7.5+1 ^{0}C for complete spawn run and appearance of first flush respectively.

Viziteu (2000) stated that seasonal variations seriously affected the number, weight and crop production period of oyster mushrooms. They reported that favourable temperature and moisture condition enhanced the production of fruiting bodies of mushrooms. Sumi (2016) conducted multilocation trials of *Hypsizygus ulmarius*, in Idukki, Wayanad and Thiruvananthapuram of Kerala. The study revealed that the mushroom can be cultivated throughout the year except April-May, in the cool climate of Idukki and Wayanad. She stated that environmental factors highly influenced the production of sporocarps and its maximum production was recorded during October-January.

2.14. DEVELOPMENTAL MORPHOLOGY

Spawn run period or incubation period is the process that allows the colonization of the substrate by mushroom mycelia and it is the first phase of mushroom cultivation cycle. It varies with the species and also depends on the environmental conditions (Higaki *et al.*, 2000).

The formation of primordia or sporophore initials of about one mm diameter and one mm height indicates the beginning of sporophore development or fruiting phase (Wood, 1976). Formation of primordia can be stimulated by abrupt changes in temperature, humidity, gas concentration, light, nutrient

reserves and physical stimuli. Under optimum ecological condition, primordia expand to reach its biological maturity (Zadrazil, 1978). Quimio (1986) reported that, *P. cystidiosus* needed 5-6 weeks for the complete colonization of 1.2 kg bag containing sawdust-rice bran substrate when spawned with 10 g of sawdust spawn. *P. cystidiosus* took 12-16 days for spawn run, 4-5 days for primordia formation and 4-8 days for fruiting body development with a cropping cycle of 30 days giving two crops at an interval of 10 days apart (Stamets, 1993). Mshandete and Cuff (2008) found that *P. flabellatus* took 16-18 days for the first appearance of mushrooms on composted sisal decortications residue with a BE of 28 per cent. Lechner *et al.* (2011) reported that *P. albidus*, *P. djamor*, *P. ostreatus*, *P. pulmonarius* and *P. cystidiosus* required 20 to 42, 27 to 37, 25 to 40, 28 to 42 and 45 days respectively for primordia development after spawning. Kalaw and Albinto (2014) reported that *P. cystidiosus* took 43.05 and 48.77 days on rice straw + sawdust, whereas, *P. florida* took 19.13 and 21.9 days for spawn run and pinhead formation respectively, with BE of 72.70 and 28.87 per cent. *P. djamor* required a spawn running period of 18-19 days, and 6-7 more days for first harvest with a cropping period ranging from 18-32 days (Pandey *et al.*, 2016). *P. eous* could be harvested from the 12th day of bed preparation and continued up to 26th day with a total of three harvests (Shoba and Krishnakumari, 2017).

2.15. PESTS AND DISEASES

Spoilage of spawns due to fungal and bacterial contaminants is one of the biggest constraints faced by the mushroom growers. Mazumder *et al.* (2005) identified eight fungal contaminants *viz.*, *Aspergillus flavus var. cotumneris, Aspergillus niger, Alternaria alternata, Penicillium janthinellum, Penicillium* spp., *Rhizopus stotoniter, Trichoderma harzianum, Trichoderma viride* and one bacterial contaminant *viz., Bacillus brevis*, from contaminated spawns of *P. ostreatus*. Paddy grain based spawn recorded the lowest (15 per cent contamination when compared to wheat grain based spawn (30 per cent) with highest contamination during the monsoon season (28.57 per cent) followed by pre-monsoon (21.90 per cent).

Lim *et al.* (2008) isolated eight distinct bacterial genera *viz.*, *Bacillus*, *Enterobacter*, *Sphingomonas*, *Staphylococcus* and *Moraxella* from the diseased mycelia of *P. eryngii*. Mejia and Alberto (2013) stated that when contaminants *viz.*, *Trichoderma* spp. and *Aspergillus* sp., were scarce in the substrate, they couldn't compete with the mycelium of *Pleurotus,* which could quickly colonize the substrate. Qiu *et al.* (2017) isolated *Trichoderma asperellum* from contaminated substrate of *P. ostreatus* with green mold disease. They found a positive correlation between high temperature and colonization of mushroom mycelia by *T. asperellum*. Kumar and Sarathi (2017) recorded competitor moulds *viz.*, *T. viride, T. harzianum, Penicillium notatum, A. niger, A. flavus, Mucor sp., Rhizopus sp., Sclerotium rolfsii and Coprinus spp.* in the cultivation of *P. florida*. They revealed that 0.01g of Carbendazim 50 per cent WP + 0.15 ml of formalin/100 ml showed maximum inhibitory effect on the competitor molds. *Azadirachta indica* exhibited maximum inhibitory effect on *Aspergillus* spp., *Trichoderma* spp., *Coprinus* spp., and *Penicillium* spp. (54.1 to 71.6 per cent), with minimum effect on the mycelial growth of *P. ostreatus*, followed by the extract of *Pongamia pinnata* (Biswas, 2015). Cultivated mushrooms are subject to attack by a variety of pests, especially during warm weather. Singh and Sharma (2016) documented major insect pests of mushroom *viz.*, hump backed flies (Phorids), midges (Cecids), mites (Tarsonemid-mites, Red pepper/ Pygmy mites and Tyroglyphid mites), springtails and beetles.

2.16. PROXIMATE CONSTITUENTS

Oyster mushrooms are rich in vitamins such as thiamine (4.8 mg), riboflavin (4.7 mg) and niacin (108.7 mg), minerals like; calcium (98 mg), phosphorus (476 mg), ferrous (8.5 mg) and sodium (61 mg) on 100 g dry weight basis (Pandey and Ghosh, 1996). The chemical composition of edible mushroom determines their nutritional value and sensory properties that differs according to species but also varies as per the substratum, atmospheric conditions, age and part of the fructification (Manzi *et al.*, 2004). Since the first report of hypotensive

activity of the *Pleurotus* mushrooms, these are classified as 'mushroom nutraceuticals' (Patel *et al.*, 2012).

2.16.1. Moisture

Fresh fruiting bodies of *Pleurotus* spp. contain 85-90 per cent moisture (Khan and Tania, 2012) and the moisture percentage depends on the mushroom species besides other parameters related to harvest, growth, culinary and storage conditions (Reis *et al.*, 2012).

Dunkwal *et al.* (2006) reported moisture content of 86.1 to 90.6 per cent in *P. sajor-caju* and *P. florida*. *P. high-king*, *P. ostreatus and P. geesteranus* contained 87.8, 89.4 and 86.2 per cent moisture respectively on fresh weight basis (Ahmed *et al.*, 2013). *P. eous, P. florida, P. sajor-caju, P. djamor*, *P. ferulae*, *P. sapidus*, *P. flabellatus*, *P. eryngii* and *P. ostreatus* contained moisture contents of 92.2, 91.5, 90.1, 82.21, 91.11, 90.53, 90.95, 93.09 and 90.8 per cent respectively on fresh weight basis (Maftoun *et al.*, 2015). Shevale and Deshmukh (2016) recorded the moisture content of *P. citrinopileatus, P. florida* and *P. sajar caju* as 90, 90.8 and 87.84 per cent respectively on fresh weight basis.

2.16.2. Carbohydrates

Carbohydrate is the major constituent in *Pleurotus* species, ranging from 46.6-81.81per cent (Bano and Rajarathnam, 1982). Carbohydrate compounds were present in a variety of polysaccharides with different particle sizes *viz.*, glycogen and indigestible forms as dietary fibre, cellulose, chitin , mannans and glucans (Manzi and Pizzoferrato, 2000).

Bano and Rajarathnam (1982) reported 40.64 to 55.92 per cent total carbohydrate content in *P. cystidiosus*. Patil *et al.* (2010) reported that carbohydrate content of *P. cystidiosus* ranged from 6.30 to 7.57 per cent. Carbohydrate in *Pleurotus* spp. are mainly in the form of polysaccharides, most abundant being chitin, α, β-glucans, and hemicelluloses. The polysaccharides

ranged from 36 to 60 g 100 g^{-1} dry weight of the fruiting bodies (Khan and Tania, 2012). Carbohydrate content of *P. djamor* (Shimoga isolate), pink *Pleurotus* spp. (Balaghat isolate), white *Pleurotus* spp. (Balaghat isolate) and *P. cystidiosus* (Bangalore isolate) were 50.59, 51.41, 53.22 and 33.43 per cent respectively on dry weight basis (Pandey and Veena, 2012). Total soluble sugars, reducing sugars and non-reducing sugars were 3.01, 0.38 and 2.63 per cent respectively *P. florida* (Maftoun *et al.*, 2015).

2.16.2.1. Polysaccharide

The stimulation of host's defence by bioactive polysaccharides derived from medicinal mushrooms has a significant effect on the maturation, differentiation and proliferation of many types of immune cells in the host (Wasser, 2011). Polysaccharides (including glucans) are the most bioactive, mushroom-derived, antitumor/immunomodulating compound (Giavasis, 2014).

Li *et al.* (2007) isolated a polysaccharide complex from fruiting bodies of *P. abalonus* which prolonged the lifespan of senescence-accelerated mice by the up-regulation of antioxidant genes. Atri *et al.* (2012) identified three main sugars *viz.*, sucrose (0.338–2.011 per cent), glucose (0.553-0.791 per cent) and xylose (0.01 per cent) when fruiting bodies of *Pleurotus spp* were analysed. Silva *et al.* (2012) isolated, purified and characterized polysaccharides *viz.*, intracellular ($0.013 \pm 8.12\times 10^{-5}$ g L^{-1} day^{-1}) and extracellular polysaccharides (0.037 ± 0.0005g L^{-1} day^{-1}) from submerged culture of *P. ostreatus*. Yang *et al.* (2013) isolated a polysaccharide named PEPw from the fruiting bodies of *P. eryngii,* which improved the cellular immunity in Renca tumor-bearing mice and inhibited the tumor growth of Renca renal cancer in mice. *P. cystidiosus* is rich in glucose (11.6 mg g^{-1}), mannitol (24.6 mg g^{-1}) and trehalose (28.6 mg g^{-1}), with a total sugar content of 64.9 mg g^{-1} (Maftoun *et al.*, 2015). Polysaccharides have been isolated from *P. sajor-caju* (Telles *et al.*, 2011), *P. eous* (Xu *et al.*, 2015) and *P. eryngii* (Abdullah *et al.*, 2017).

2.16.3. Protein

Mshandete and Cuff (2008) stated that protein content of edible mushrooms is influenced by species/strain and the growing substrate. Protein content in mushrooms is ranked above vegetables, fruits and milk (Chang, 2008).

The protein content in *Pleurotus* species varied from 8.9 to 38.7 per cent on dry weight basis and contains all essential aminoacids making them a substitute for meat diet (Kurtzman, 2005). Alam *et al.* (2008) identified the protein content (g 100 g^{-1} dried matter) of *P. ostreatus* (23.91g), *P. sajor-caju* (24.63 g), *P. florida* (20.56 g) and *C. indica* (21.4 g). Protein content of *P. djamor* (Shimoga isolate), pink *Pleurotus* spp. (Balaghat isolate), white *Pleurotus* spp. (Balaghat isolate), *P. cystidiosus* (Bangalore isolate) and white *Pleurotus* spp. (Madurai isolate) were 29.81, 30.87, 24.56, 24.68 and 36.25 per cent, on dry weight basis respectively (Pandey and Veena, 2012). *P. cystidiosus* is rich in amino acids *viz.*, isoleucine, valine, tryptophan, lysine, thereonine, phenyalanine, cysteine, aspartic acid, serine, glutamic acid, proline, glycine, arginine and tyrosine (Maftoun *et al.*, 2015). Mishra *et al.* (2015) reported that *H. ulmarius* has higher protein content (33.6 per cent) followed by *P. florida* (33.5 per cent), *P. sapidus* (32.5 per cent) *P. citrinopileatus* (32.3 per cent), *P. djamor* (30.6 per cent), *P. flabellatus* (30.3 per cent) and *P. platypus* (28.3 per cent).

2.16.4. Fat

Fat content in mushrooms is very low compared to carbohydrates and proteins, hence recommended for low calorie diets. In general, the crude fat of mushrooms has representatives of all classes of lipid compounds including free fatty acids, monoglycerides, diglycerides triglycerides, sterols, sterol esters and phospholipids. Fat content in *Pleurotus* species ranged from 1.08 to 9.4 per cent (Food and Nutrition Board, 2001). Ali *et al.* (2007) recorded the ash content in *P. ostreatus*, *P. sajor-caju*, *P. florida* and *P. corunucopiae* as 2.15, 2.21, 3.38 and 2.83 per cent, respectively, when rubber sawdust was used as substrate. Fat content of *P. djamor* (Shimoga isolate), pink *Pleurotus* spp. (Balaghat isolate),

white *Pleurotus* spp. (Balaghat isolate) and *P. cystidiosus* (Bangalore isolate) were 4.5, 5.1, 4.9 and 5.04 per cent, on dry weight basis respectively (Pandey and Veena, 2012). Atri *et al.* (2013) reported that, among the fatty acids, the monounsaturated ones are present in a higher proportion (37.17-68.29 per cent) than the saturated ones (26.07-47.77 per cent) in *Pleurotus* spp. Fat content of 1.7 and 0.48-0.91 per cent was recorded in *P. sajor-caju* and *P. florida,* respectively (Salami *et al.,* 2017).

2.16.5. Fibre

Mushrooms are a potential source of dietary fibre due to the presence of non-starch polysaccharides. Total dietary fibre in mushrooms is composed of intrinsic non-digestible carbohydrates, mainly chitin and glucans are the important components of soluble or insoluble dietary fibres (Vetter, 2007).

Goyal and Grewal (2006) determined, the total dietary fibre (sum of hemicelluloses, cellulose and lignin content) of *P. sajor caju* as 43.73 per cent. Fresh fruiting bodies of *P. sajor-caju, P. florida*, *C. indica* and *P. ostreatus* contained 2.8 to 3.1 g, 2.9 to 3.1 g, 1.5 to 1.8 g and 3.2 to 3.6 g of fiber per 100 g (Alam *et al.*, 2008). Khan *et al.* (2008) recorded the fibre content in *P. sajor-caju* (26.2 per cent), *P. ostreatus* (27 per cent), *P. florida* (26.8 per cent), *P. cystidiosus* (25.5 per cent) and *P. geestaranus* (26.3 per cent). *P. djamor*, pink *Pleurotus*, white *Pleurotus* spp and *P. cystidiosus* contained 13.2, 27.8, 5.1 and 4.9 per cent crude fibre on dry weight basis respectively (Pandey and Veena, 2012). Bora and Kawatra (2014) recorded the acid detergent and neutral detergent fibre content of *P. florida* as 17.82 and 43.22 g per 100g, respectively.

2.16.6. Ash

Ash content represents the total amount of mineral content present in the mushroom and it varies significantly among different *Pleurotus* species. Manzi *et al.* (2004) reported that oyster mushrooms contain 3-3.80g/100g of ash. Alam *et al.* (2008) recorded, the ash content in *P. florida*, *P. ostreatus* and *C. indica* as 6.6,

7.3 and 13.1 per cent, respectively. *P. high-king*, *P. ostreatus* and *P. geesteranus* contained 11.5, 8.6 and 12.8 per cent ash, respectively (Ahmed *et al.*, 2013). Deepalakshmi and Mriunalini (2014) recorded the ash content in *P. ostreatus* as 0.8g/100 g. Wild oyster mushroom collected from North-Eastern Part of Uttar Pradesh *viz.*, *P. flabellatus*, *P. florida* and *P. ostreatus* have 5.72, 6.44 and 7.69 per cent ash respectively (Vishwakarma *et al.*, 2017). Ash contents of 6.1, 11.06 and 5.18-6.39 per cent was recorded for *P. sajor-caju*, *P. florida* (Salami *et al.*, 2017) and *P. ostreatus* (Tolera and Abe, 2017).

2.16.7. Total antioxidants

Mushrooms are rich in antioxidants which can reduce the oxidative damage in humans, thus preventing diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Yang *et al.*, 2002). Antioxidant properties of mushrooms are related to the presence of phenolic compounds, ascorbic acid, carotenoids, tocopherol, vitamin C and selenium (Mau *et al.*, 2005).

Methanolic extracts of *P. ostreatus* and *P. cystidiosus* fruiting bodies possessed antioxidant/reducing, radical scavenging and iron chelating activities, higher than the other commercial mushrooms (Yang *et al.*, 2002). DPPH radical scavenging activity of *P. ferulae* was recorded as 9 mg mL^{-1} (Kim *et al.*, 2012). Hexane and dichloromethane extracts of *P.* showed DPPH radical scavenging activity with EC50 values of 0.81 and 0.82 mg/ml respectively (Menikpurage *et al.*, 2012). Methanol extract of *P. florida* fruiting body showed DPPH radical scavenging, with IC50 value of 50 µg ml^{-1} (Menaga *et al.*, 2013). Methanolic extract of *P. eous* recorded antioxidant capacity of 88 mg equivalents of butylated hydroxytoluene (BHT)/100 mg of extract (Suseem and Saral, 2013). Babu *et al.* (2014) reported that, ethanolic extracts of *P. florida, P. sajor-caju, P. cystidiosus* and *P. djamor* showed good scavenging effects (73.3 to 42.4 per cent) on DPPH radicals at a concentration of 1.5 mg/ml. *P. cystidiosus* registered higher DPPH free radical scavenging activity (72.97 per cent) compared to *Coprinus comatus* (91.90 per cent) (Kalaw and Albinto, 2014).

Muruke (2014) reported that irrespective of the preservation method and portion of the mushroom used, *P. cystidiosus* exhibited very high number of antiradical activity units in 1 mg of extract *i.e.* EAU 515 value, ranging from 3.25 to 17.00. *P. cystidiosus* also portrayed very high DPPH free radical scavenging ability at very low concentration of crude extracts (0.0125 to 0.800 mg/ml), with EC50 values ranging from 0.035 to 0.150 mg ml^{-1}. Prabu and Kumuthakalavalli (2016) recorded the IC50 value of *P. florida* as 413.28 µg ml^{-1}.

2.16.8. Beta carotene

Beta carotene is a member of carotenoid family, which are highly pigmented (red, orange, yellow), fat-soluble compounds naturally present in fruits, grains, oils and vegetables. Beta carotene is known as 'provitamin A carotenoids', because these can be converted to vitamin A in the human body (Vishwakarma *et al.*, 2017).

Wild collections of *P. floridanus*, *P. pulmonarius*, *P. sapidus*, *P. cystidiosus* and *P. sajor-caju*, from Northwest India, contained β-carotene ranging from 0.134 to 0.221 µg/100 g (Atri *et al.*, 2013). *P. flabellatus* has beta carotene content of 7.17ng/mg (Dasgupta *et al.*, 2013). Obodai *et al.* (2014) reported that beta carotene content of *P. pulmonarius, P. ostreatus, P. sapidus* and *P. citrinopileatus*, varied from 0.60 to 11.46 µg g^{-1}. *P. cystidiosus Agaricus campestris*, *Agaricus arvensis* and *Amanita caesarea* has a β-carotene content of 0.79, 0.50, 0.75 and 0.71 ug $100g^{-1}$, respectively (Sharma and Gautam, 2015). *P. cystidiosus*, *P. flabellatus*, *P. florida* and *P. ostreatus* contained β-carotene contents of 10, 12, 6.5 and 0.7 µg mg^{-1}, respectively (Vishwakarma *et al.*, 2017).

2.16.9. Polyphenols

Phenolic acids predominate the phenolic profile of edible mushrooms, but some species also contain varying amounts of catechin and myricetin (Palacios *et al.*, 2011). Hydroxyl groups of phenolic compounds are responsible

for the scavenging ability, contributing directly to the antioxidant action of these compounds (Paterson, 2006).

Dubost *et al.* (2007) revealed that, 85 g of button mushroom contains 43-75 mg of total phenols. He also found a positive correlation between the high phenol content and free radical sequestering ability. Adefegha and Oboh (2011) and Tibuhwa (2012) reported high phenolic contents in oyster and termitarian mushrooms. Methanolic extracts of *P. eous* (354.59 mg gm^{-1} of dry mass) recorded the maximum phenol content, followed by ethyl acetate (189.37 mg gm^{-1} of dry mass) and petroleum ether extract (146.38 mg gm^{-1} of dry mass) (Suseem and Saral, 2013). Total phenolic content of methanolic extracts of *P. florida, P. sajor-caju, P. cystidiosus* and *P. djamor* ranged from 22.67 to 36.03 mg g^{-1} (Babu *et al.*, 2014). Kalaw and Albinto (2014) identified a total phenolic concentration of 3.41 and 17.82 mg Gallic acid equivalent g^{-1}, for *P. cystidiosus* and *Coprinus comatus*, respectively. Total phenolic contents and IC50 values of *P. pulmonarius, P. ostreatus, P. sapidus* and *P. citrinopileatus* ranged from 1.48 to 3.58 µg of GAE (gallic acid equivalents) g^{-1} and 43.21 to 52.03 µg ml^{-1}, respectively (Obodai *et al.*, 2014). Sharma and Gautam (2015) found that *P. cystidiosus* contained phenolic compounds of about, 53.20 mg 100 g^{-1} of gallic acid.

2.16.10. Energy value

Energy value is derived from the oxidation of carbohydrates, proteins and fats. Khan *et al.* (2008) recorded the energy value of *P. florida* and *P. cystidiosus* respectively as 250.1 Kcal and 262.8 Kcal per 100 g of dry sample. Energy value of *P. ostreatus* is recorded as 1510 KJ kg^{-1}, making it suitable for inclusion into calorie-controlled diets (Jaworska and Bernas, 2011). Total energy contribution of *P. cystidiosus* and *P. florida* was recorded as 287 and 304.85 Kcal 100 g^{-1} dry weight, respectively (Hoa *et al.*, 2015). Maftoun *et al.* (2015) recorded the energy values of *P. eryngii* and *P. ostreatus* as 421 and 350 Kcal $100g^{-1}$, respectively.

Khan *et al.* (2008) recorded the proximate constituents of *P. florida* and *P. cystidiosus*, *viz.*, protein (20.6, 17.7 g $100g^{-1}$), carbohydrate (40.3,44 g $100g^{-1}$), fibre (26.8,25.5 g $100g^{-1}$), ash (8.3,7.4g $100g^{-1}$) and metabolizable energy (250.1, 262.8 Kcal $100g^{-1}$). *P. cystidiosus* is rich in all the essential nutrient component including carbohydrates (85.86 per cent), proteins (3.1 per cent), fibres (3.12 per cent) and ash (2 per cent). Besides this, it has low fat (0.8 per cent) contents (Atri *et al.*, 2012). Carbohydrate, protein, crude fat, crude fibers, ash and total phenolic content of *P. floridanus*, *P. pulmonarius*, *P. sapidus*, *P. cystidiosus* and *P. sajor-caju* (Fr.) Singer) ranged from 85.86 to 88.38 per cent, 0.98 to 2.17 per cent, 0.62 to 0.84 per cent, 2.76 to 3.12 per cent, 1.03 to 2.20 per cent and 6.76 to 16.92 mg/100 g of gallic acid respectively (Atri *et al.*, 2013). Khan *et al.* (2013) recorded protein, fat, crude fibre, ash, dry matter and moisture contents of *Pleurotus* (*flabellatus*) *djamor* as, 21.34, 0.41, 8.17,6.47,6.13 and 79.89 per cent respectively. *P. florida* contained 91.80, 27.92, 7.82, 0.72, 11.87 and 47.80 per cent moisture, crude protein, total ash, fat, crude fibre and carbohydrates respectively (Bora and Kawatra, 2014). Kalaw and Albinto (2014) recorded that, *P. cystidiosus* contains, 17.47, 13.42, 1.4, 13.42, 1.4, 13.62 and 48.39 per cent of protein, crude fibre, fat, ash and carbohydrates, respectively with energy value of 268.62 Kcal $100g^{-1}$.

Hoa *et al.* (2015) found that fat and fibre content of *P. cystidiosus* ranged from 2.05-3.33 and 20.05 to 25.05 per cent, respectively, on dry weight basis. Sharma and Gautam (2015) recorded, protein, crude fat, fibre, ash and carbohydrate content of *P. cystidious* as 20.69, 0.20, 2.16, 0.42, 52.20 per cent, respectively, with DPPH radical scavenging activity of 2.17 mg ml^{-1}. Kwagyan *et al.* (2016) found that *P. eous* contain 83.44 to 88.98, 6.53 to 10.54, 14.87 to 19.05, 9.35 to 28.6 and 21.63 to 35.99 per cent dry matter, ash, fat, carbohydrate and protein contents on dry weight basis. Vishwakarma *et al.* (2017) recorded protein, carbohydrate, lipid, moisture, ash and fibre contents of *P. cystidiosus* as, 41.11, 40.49, 0.42, 85.18, 6.65 and 17.55 per cent, respectively.

2.17. ANTICANCEROUS ACTIVITIES

Cancer has become the second largest cause of death in humans and has led to many research efforts and clinical studies to find potent cancer drugs (Daba and Ezeronye, 2003). Treatments using chemotherapeutic agents, surgery and radiation have not been fully effective in the control or prevention of many cancers (Moongkarndi *et al.*, 2004). In this context, mushroom is one of the most important functional food with many therapeutic applications including chemotherapy (El-Enshasy and Kaul, 2013). Investigators have identified antitumor compounds of mushroom origin such as polysaccharides, lentinan, β-glucans, lucidenic acid, ergosterol, proteins, proteoglycans and deoxy-ribonucleic acid (DNA) (Telles *et al.*, 2011; Xu *et al.*, 2015).

Extract of *P. ostreatus* mycelium alone or in combination with chemotherapeutic agent, cyclophosphamide, inhibited the *in-vivo* tumour growth in mice (Meerovich *et al.*, 2005). Ajith and Janardhanan (2007) identified the antioxidant and antitumor activities of extracts of *Ganoderma lucidum*, *Phellinus rimosus*, *P. florida* and *P. pulmonaris*. Kudahewa *et al.* (2008) identified that *P. cystidiosus* fractions *viz.*, A4-2 and A4-3 demonstrated high antioxidant and anti-proliferative activities. Polysaccharides isolated from *P. abalonus* showed anti-proliferative effects in breast (MCF7) and liver carcinoma (HepG2) cells (Wang *et al.* 2011). Menikpurage *et al.* (2012) studied cytotoxicity of *P. cystidiosus* against Hep-2 cancer cells using 3[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) (MTT) assay. They found that cancer cells incubated with 5 mg ml^{-1} of the extract, showed morphological changes such as cellular swelling, irregular cell shapes, condensed cytoplasm and vacuolar areas. Ren *et al.* (2015) isolated a heteropolysaccharide named PAP, from *P. abalonus*. PAP exhibited strong free radicals-scavenging, antioxidant and antiproliferative effect against LoVo cancer cell line. A sesquiterpenoid named, pleuroton B, extracted from *P. cystidiosus* induced apoptosis in two human prostate cell lines (DU-145 and C42B) (Zheng *et al.,* 2015). Aqueous extract of polysaccharides from the fruiting body and mycelium of *Pleurotus tuber-regium* exhibited strong

cytotoxicity (IC50 value of 25 µg/mL) as well as effective anti-proliferative activitiy (200 µg/mL) against human acute promyelocytic leukemia cells (HL-60) (Sharma *et al.*, 2016).

2.18. KEEPING QUALITY

Mushrooms of *Pleurotus* genus are delicate and sensitive, and start deteriorating within one day after harvest. Development of brown colour due to enzymatic action is the first sign of deterioration and is a major factor contributing to quality losses (Apati *et al.*, 2010).

YounMoon *et al.* (2004) recorded the maximum shipping period of oyster mushroom as 21 days at a low temperature of 7 ^{0}C, while stored in tray type packages. Pandey and Veena (2012) found that post harvest storage of *P. djamor* in trays wrapped with antifog film at 27-29^0 C and 4-6^{0}C recorded shelf life of 1-2 and 4-6 days respectively, depending on the harvesting stage. The colour of sporophore faded faster on storage. Also, no aerial hyphae was visible on the sporophores even after 48 h. Post harvest storage of *P. cystidiosus* in trays wrapped with antifog film at 27-29 ^{0}C and 4-6 ^{0}C recorded shelf life of 3-4 days and 20-30 days respectively. They also found that, black sporophores showed better shelf life than lighter colored sporophores without the formation of aerial hyphae for 3 to 4 days.

2.19. SENSORY EVALUATION OF FRUIT BODIES

Abbott (1999) stated that quality implies the degree of excellence of a product or its suitability for a particular use. It comprises the sensory properties *viz.*, appearance, taste, flavour and mouth feel (texture). Sensory analysis provides an index of overall acceptability of food stuffs which depends on these properties, thus determining its commercial viability. The organoleptic qualities and product acceptability can be easily identified using a consumer oriented approach even though these attributes cannot be accurately and precisely measured (Gathambiri *et al.*, 2009). Taste of mushrooms is influenced by the presence of sweet

components (alarnine, glycine and threonine), monosodium glutamate like compounds and bitter compounds high in phenolic compounds (Chen, 1986). Juan-Hwan *et al.* (2001) identified that abalone mushroom has relatively higher levels of sweet compounds which mask the bitterness and contributed to the significantly higher overall acceptability of *P. abalonus* than that of *P. florida.* The terpenes, lactones, amino acids, and carbohydrates of fruiting bodies determined aroma and flavour characteristics to their fruiting body and mycelial biomass (Smiderle *et al.*, 2008). Munoz and Amaya (2013) recorded the texture of *P. opuntiae* as compact and fibrous meat with a mild taste and fungal odour. *P. opuntiae* is delicate to eat at the young stage whereas, it turns tough and loses its taste as it mature. Gateri *et al.* (2015) made organoleptic scores according to a five point hedonic scale and stated that *P. abalonus* was more superior to *P. florida* in terms of appearance, taste, flavour and overall acceptability.

Value added products prepared from mushrooms not only reduces the post harvest losses but also provide neutraceutical low fat and protein rich food to the consumers. Jaziya (2011) reported that incorporation of oyster mushroom to dishes, was very acceptable to the respondents. *P. opuntiae* have been traditionally used as a food and remedy for several health problems and also as a traditional alcoholic drink from *Agave* (Camacho *et al.,* 2012). Okafor *et al.* (2012) found that supplementation of wheat flour with 25 per cent *P. eous* powder improved the bread making properties, proximate composition and sensory qualities. Bora and Kawatra (2014) rated value added products prepared from *P. florida* based on a 9 point Hedonic scale and found that, mushroom preserve (murraba) rated the highest sensory score followed by mushroom chips, mushroom ketch-up and mushroom candy. Tan *et al.* (2015) found that pressure cooking increased the free radical scavenging abilities of *P. floridanus*, *P. flabellatus* and *P. pulmonarius* by 200, 117.6 and 49.1 per cent, respectively, compared to the uncooked samples, whereas *P. cystidiosus* showed a significant decrease in the antioxidant activity by 63 per cent.

Mushroom based new products and fortified mushroom products have been developed including mushroom fortified corn extrudates, fortified cakes, ready to cook frozen mushroom tikki. Fortification levels of mushroom in the extrudates were optimized as 20 per cent paste and 10 per cent mushroom powder for both single and twin screw extruders (ICAR, 2015). Mushroom powder have been used by many researchers for development of variety of food products *viz.*, cookies and breads (Regula and Michalowska, 2010); biscuits (Wakchaure *et al.*, 2010); jam and squash (Lakshmipathy *et al.*, 2013) and rava idli (Paul, 2016).

2.20. POST HARVEST TECHNOLOGY

Poor postharvest storage conditions provoke changes in the physiological and molecular mechanisms of mushrooms because they have no cuticle to protect from physical, microbial attack or water loss. Browning, veil opening, weight loss, microbial spoilage, changes in polysaccharides composition and polyphenol oxidase reaction are the common postharvest changes in mushrooms which often result in enormous economic losses (Banfi *et al.* 2015). In the past years, diverse post harvest treatments have been investigated in an effort to discover new alternatives for extending the mushroom shelf life *viz.*, cold storage (Dama *et al.*, 2010), modified atmosphere packaging (MAP) (Guillaume *et al.*, 2010), gamma and electron beam irradiation (Fernandes *et al.*, 2012) and coating treatments (Jianglian and Shaoying, 2013).

Drying is a classical method of food preservation based on the principle that, water activity of the product is lowered to defined levels that guarantee the microbiological and physicochemical stability (Lewicki and Jakubczyk, 2004). Sun-drying is the oldest method of preservation of mushroom and is carried out in days with high temperature and low humidity. Sun dried mushrooms can retain 3-4 per cent of moisture (Desayi, 2012). Lakshmipathy *et al.* (2013) stated that solar drying proved to be more effective than oven drying as the former recorded higher overall acceptability of powders.

Jandaik and Sharma (1983) reported that mechanical drying of mushrooms, at 45-50^{0}C was better than drying at 55-60^{0}C as in former case sporophores retained only 29 per cent moisture, with acceptable organoleptic taste and slight browning, whereas drying at 55-60^{0}C resulted in 100 per cent moisture free sporophores with organoleptically non-acceptable burnt taste. Beelman (1987) recorded that dehydration (sun drying) and dehydrated cum powering recorded, the highest score for colour, odour, appearance and taste (score of over 4 out of 6) followed by canning (score of about 3). He also identified treatments *viz.*, sugar+vinegar, vinegar, sugar, blanching+vinegar, blanching+sugar resulted in poor storage life. *P. ostreatus and P. cystidiosus* showed improved flavonoid content and antioxidant properties with freeze-drying method compared to oven-drying method (Hoa *et al.*, 2017).Washing mushrooms with anti-microbial and anti-browning agents have recently gained commercial popularity as a means of extending the shelf life of mushrooms (Singh *et al.*, 2016).

Pretreatments of mushrooms prior to drying *viz*, washing in 0.5 per cent calcium chloride and 0.5 per cent citric acid (Gothandapani *et al.*, 1997), combination wash with 50 ppm chlorine dioxide, 0.1 per cent sodium erythrobate and 0.05 per cent calcium chloride (Singh *et al.,* 2007), and 0.5-1.5 per cent potassium metabisulphite (Jayathunge and Illeperuma, 2005), either alone or in combination, helped in checking enzymatic browning, maintaining colour, improving flavour retention and retaining the textural properties. Hassan and Ghada (2014) reported that drying time ranged between 7- 12 h and 6.5-11 h for *P. ostreatus* and *P. eryngii*, respectively for all pretreatments. They found that among pretreatmens, blanching took more drying time compared to control and steeping.

Citric acid is mainly used in the food industries as a preservative, due to its antioxidant, pH decreasing and metal chelating action (Martine *et al.,* 2000). Soaking of whole oyster mushrooms in 40 g L^{-1} of citric acid or 50 ml L^{-1} of H_2O_2 followed by slicing, increased the shelf life from 11 days to 15 and 14 days

respectively at 4 ^{0}C (Brennan *et al.*, 2000). Simon and Fandos (2010) stated that citric acid reduced the number of pseudomonas bacteria by the dissociation of acid molecule within bacterial cells and chelation of metal ions necessary for bacterial growth Khan (2015) reported that two minute dips in citric acid combined with calcium chloride and sorbitol retained the antioxidant enzymes in stored mushrooms. Gupta and Bhat (2016) found that, 2.5 per cent citric acid was the most effective treatment in controlling weight loss, maturity index and microbial growth up to a period of 12 days and was found to be the most organically acceptable.

Mohamed and Hoo (1994) documented that sulphites retard both enzymatic and nonenzymatic browning besides, exerting a bleaching action on pigments present. The optimum temperature for drying of sulphited mushroom was 60^{0}C to retain the original colour and flavour of mushrooms (Dang and Singh, 1978). Improvement in colour of mushrooms after sulphitation has been reported by many workers *viz*. Deshpande and Tamhane (1981), Mudahar and Bains (1982) and Suguna *et al.* (1995). Storage of mushrooms after treatment with the preservative, potassium metabisulphite (KMS) at 1.5 per cent reduced the microbial spoilage (Gothandapani *et al.*, 1997). Simon and Fandos (2010) recorded that combined treatment of citric acid with an anti-browning agent *viz*., 1.5 per cent sodium ascorbate, resulted in a 2.8 log reduction in the count of *Pseudomonas* bacteria. Ndabikunze *et al.* (2011) reported that microbial loads were significantly reduced to 4.3 log CFU ml^{-1} in mushrooms treated with KMS. When *P. florida* was subjected to various pre-treatments, total mesophilic and psychrophilic bacteria gradually increased during the storage period. Kumar *et al.* (2013) found that pretreatment of mushrooms with one per cent KMS, folllowed by drying in medium size dryer gave the maximum values of whiteness, rehydration ratio and coefficient of rehydration. Fan *et al.* (2017) revealed that count of mesophilic bacteria was higher than psychrophilic bacteria, because most of the microbes grow and reproduce under moderate conditions. Wakchaure (2017) reported that washing of mushrooms in 0.05 per cent potassium

metabisulphite improved the initial whiteness of *Agaricus bisporous* which lasted during the storage. Also, steeping of blanched mushrooms in one per cent KMS along with two per cent citric acid for 12 h followed by drying improved the color, texture and reconstitution properties.

2.21. STRAIN IMPROVEMENT

Modern techniques of genetic engineering and biotechnology which have been difficult to achieve in mushroom breeding due to incompatibility barriers between strains, lack of linkage maps to localize genes of interest and by the presence of contradictory data regarding the size and organization of the genome. Somatic hybridization through protoplast fusion is one of the best way to combine genetic characters across the species barrier provided an optimized enzyme combination is available to give a good score of purified protoplasts. Hybrid protoplasts so developed contain heteroplasmic cytoplasm and fused parent nuclei (Murlidhar and Panda, 2000).

2.21.1. Protoplast isolation and purification

Protoplast is an organized body of a species with its cell wall completely or partially removed using mechanical or enzymatic means (Peberdy and Gibson, 1971).

2.21.1.1. Barrage reaction

Fischer and Bresinsky (1992) showed that compatible reactions of hyphal fusions in *Phellinus torulosus* resulted in the formation of heterokarotic mycelium in the contact zone, followed by border zone becoming unrecognizable through the time. Positive compatible reactions have been reported between *P. ostreatus* and *P. cornucopias* (Esser and Blaich, 1994), *P. pulmonarius* and *P. florida* (Eyini *et al.*, 2006); and *P. pulmonarius* and *P. citrinopileatus* (Rosnina, 2017).

2.21.1.2. Selection by biochemical markers

Biochemical markers can reflect the genetic study because they are direct product of genes (Chaudhary and John, 2017).

Singh *et al.* (2007) used 210 µg ml^{-1} griseofulvin and 10 µg ml^{-1} clotrimazole as markers for the development of fusants between *A. bisporus* and *A. bitorquis*. Naseema and Elizabeth (2010) used lead acetate (4500 ppm) and Nizral (2000 ppm) as dual biochemical markers for the selection of fusion product between *F. oxysporum* and *F. pallidoroseum. Fusarium* spp. were resistant to lead acetate (4500 ppm) and Nizral (2000 ppm), respectively and the fusants were selected by growing in PDA media amended with the dual markers. Kaur and Kapoor (2014) used 2 m*M* vanillin and 1000 ppm carbendazim as screening markers for the selection of somatic hybrids between *P. florida* and *P. sajor-caju*. Mallick and Sikdar (2014) selected nine inter-generic somatic hybrids between *P. florida* and *Lentinula edodes*, using high salt tolerance (0.7 *M* NaCl) property of *P. florida* and low salt tolerance (0.2 *M* NaCl) property of *L. edodes*. Mallick and Sikdar (2015) inactivated protoplasts of *P. florida and Lentinus squarrosulus by* 10 mM iodoacetamide and heat treatment, respectively. Since, both the parental protoplasts were inactivated, they could not grow individually in the fusion medium and strategy of inactivation enabled the easy selection of fusants.

2.21.1.3. Optimization of protocol for protoplast isolation

Osmotic stabilizers play an important role in the isolation of viable protoplasts as these are osmotically sensitive. Various osmotic stabilisers such as 0.6 *M* sodium chloride (NaCl) (Hee *et al.,* 1986), 0.6 M sucrose (Rai *et al.,* 2005), 0.6 *M* potassium chloride (KCl) (Eyini *et al.,* 2006) and 0.6 M Magnesium sulphate ($MgSO_4.7H_2O$) (Mallick and Sikdar, 2014) were used for isolation of viable protoplasts.

Fungal cell wall is rich in chitin, β-glucans, α-glucan, glycoproteins and chitosan (Farkas, 1985). Enzymes that can digest the cell wall components *viz.*,

Novozyme 234, Cellulase CP, Cellulase onozuka R-10, Chitinase, β-Glucanase and snail enzyme are used for the release of protoplast from edible fungi (Peberdy, 1989). Kim *et al.,* (2010) reported that maximum protoplasts were harvested from *P. ostreatus*, *P. eryngii* and *Hypsizygus marmoreus* when homogenized mycelia was treated with the enzyme combination of Glucanex^{R}200G and cellulase onozuka R-10. Naseema and Elizabeth (2010) used 80 mg of lytic enzyme from *T. harzianum* in 4 ml 0.01 M citrate buffer (pH 5.85), containing 0.6 M KCl and 5 mM Dithiothreitol (DTT) for the isolation of protoplasts from *Fusarium pallidoroseum* and *Fusarium oxysporum*.

Efficiency of cell wall degrading enzymes depends mainly on the growing stage (mycelium age) of mushrooms. Highest number of protoplasts was derived from the youngest hyphae of *P. florida* and *P. pulmonarius* (3-day-old cultures) which yielded 5.3×10^7 and 5.8×10^7 protoplasts g^{-1} mycelium respectively (Eyini *et al*., 2006). Djajanegara and Masdukia (2010) harvested 3.15×10^5 and 3.71×10^5 protoplasts ml^{-1}, from 5-days old monokaryotic mycelia of *P. florida* and *P. cystidious,* respectively. Four day old monokaryotic mycelia of wild and mutant strains of *Pleurotus* gave protoplast yield of 3.26×10^6 and 4.56×10^6, respectively (Aswini *et al.,* 2014).

Osmotic stabilizers and media used can significantly influence the regeneration of protoplast. Eynii *et al*. (2006) noticed two types of regeneration for *Pleurotus* spp. in malt yeast glucose medium (MYG) containing 0.6 *M* sucrose, *i.e*. a chain of cells emerging from the protoplasts and a germ tube like hypha germinating from the protoplasts. Djajanegara and Masduki (2010) recorded regeneration efficiency of 80.61 and 83.68 per cent for *P. florida* and *P. cystidious* respectively when 0.6 *M* $MgSO_4.7H_2O$ dissolved in 0.01 *M* phosphate buffer and malt peptone glucose agar (MPG) were used as osmotic stabilizer and regeneration media. Parani and Eyini (2010) found that, protoplasts of *P. eous* and *P. flabellatus* recorded regeneration efficiency of 0.28 and 0.24 per cent

respectively when 0.6 *M* sucrose and PDA was used as osmotic stabiliser and regeneration medium respectively after 72 h of incubation at 30 ^{0}C.

2.21.2. Poly ethylene glycol (PEG) mediated fusion and regeneration

PEG is an effective chemical which induce protoplast aggregation and subsequent fusion. But the concentration and molecular weight of PEG are important criteria for successful fusion. Toyomasu and Mori (1987) first reported the interspecific protoplast fusion in edible mushrooms *viz*., between *P. ostreatus* and *P. salmoneo-stramineus*. Dhitaphichit and Pornsuriya (2005) developed somatic hybrids of *P. ostreatus* and *P. djamor* and standardised the parameters for protoplast isolation *viz*., agitation of mycelium at 100 rpm for 2 h with 9 mg lysing enzyme in 1 ml osmotic stabilizer (0.6 *M* $MgSO_4 \cdot 7H_2O$). They also recorded incubation of protoplasts with 40 per cent PEG 6,000 and 0.05 M calcium chloride ($CaCl_2$) for 20 min at room temperature enabled the fusion of protoplasts. Eyini *et al.* (2006) attempted inter-specific hybridization between *P. pulmonarius* and *P. florida* through PEG-induced protoplast fusion. They used mixture of mycolytic enzymes, *i.e.* commercial cellulase, crude chitinase and pectinase, 0.6 *M* KCl as osmotic stabilizer and phosphate buffer (pH 6) for the maximum release of protoplasts from 3-day-old mycelia of *P. florida* and *P. pulmonarius* with regeneration efficiency of 3.3 and 4.1 per cent respectively. PEG induced fusion recorded a fusion frequency of 0.28 per cent in MYG medium. Djajanegara and Masdukia (2010) carried out protoplast isolation and fusion between *P. florida* and *P. cystidiosus,* using a mixture of cellulase Onozuka R-10 and macerozyme R-10 for isolation and 40 per cent PEG 6000 for protoplast fusion. Parani and Eyini (2010) carried out protoplast fusion between *P. eous* and *P. flabellatus* for enhanced coffee pulp degradation. They identified that a mixture of mycolytic enzymes (cellulase, chitinase and pectinase) gave the maximum yield of protoplasts from 3 days old cultures of *P. eous* and *P. flabellatus* respectively when 0.6 *M* KCl in phosphate buffer was used as osmotic stabiliser with a fusion frequency of 0.18 per cent.

2.21.3. Confirmation and evaluation of strains/hybrids

During PEG mediated fusion, false fusants and abortive fusants may arise, which do not result in the production of heterokaryons. False fusants arise due to fusion between protoplasts of same parent. In this context, protoplast fusion and regeneration should be followed with, by selection of true fusants from the many false fusants.

2.21.3.1. Selection on the basis of colony diameter and hyphal width

Selection of hybrid fusant can be done on the basis of linear growth of mycelium on solid medium whereby, fast growing mycelium can be selected as the hybrid fusant. Liu *et al.* (2008) stated that growth rate of hypha could be considered for the characterization of the strain. Somatic fusion between *P. ostreatus* and *P. djamor* resulted in two fusants *viz.*, Fu4 and Fu5, with significant difference in colony diameter (9, 9 cm) and hyphal width (2.76, 3 µm) compared to their parents (8.83, 3.24 cm and 2.43, 2.24 µm) (Dhitaphichit and Pornsuriya, 2005). Aswini *et al.* (2014) recorded improved colony diameter (5.32 cm) and hyphal width (3.12 µm) of somatic fusant when compared to the parental strains *viz.*, wild (4.65 cm, 2.76 µm) and mutant (3.24cm, 2.43 µm)) *Pleurotus* strains. Kaur and Kapoor (2014) established the hybridity of newly developed strains from *P. florida* and *P. sajor-caju* on the basis of colony morphology, mycelial growth and hyphal traits.

2.21.3.2. Cultivation trials with protoplast isolates and fusants

Somatic hybridization between *P. pulmonarius* and *P. florida* resulted in a fusant line which produced fruiting bodies on paddy straw, but required a lower temperature of crop running (24 ± 2 ^{0}C) than its parents which could fruit at 28 ± 2^{0}C (Eynii *et al.*, 2006). Mizoguchi *et al.* (2006) isolated and regenerated protoplasts from mycelia of *P. cornucopiae*. Regenerated mycelia formed fruit-bodies without any morphological abnormalities. Chakraborti and Sikdar (2008) developed twelve somatic hybrid lines through PEG mediated fusion between *Volvariella volvacea* and *P. florida* using a double selection method. They

produced two *Pleurotus* type hybrid lines having characters of higher BE and temperature tolerance respectively. Chakraborty and Sikdar (2010) successfully generated basidiocarps from 8 out of the 14 hybrid lines, obtained from PEG mediated fusion of *P. florida* and *C. indica* var. APK2. Hybrid lines recorded increased BE, temperature tolerance, enhancement in quality of fruit body and increase in essential fatty acid *viz*., linoleic acid. Djajanegaraa and Masdukia (2010) found that fruiting bodies of fusant lines *viz*., FS1 and FS2 between *P. florida* and *P. cystidious* was not different from their parents. Fruiting bodies of FS1 were creamish white in colour identical to that of *P. floridae*, while FS2 were light brownish, intermediate to parents. Mallick and Sikdar (2014) produced a hybrid line *pfle* between *P. florida* and *L. edodes* which could produce basidiocarp on paddy straw in sub-tropical climate and showed phenotypic resemblance to *P. florida*. Mallick and Sikdar (2015) carried out somatic hybridisation between *P. florida* and *L. squarrosulus*. They developed six hybrid lines which produced basidiocarps on paddy straw substrate in sub-tropical climate.

Materials and Methods

3. MATERIALS AND METHODS

The experimental studies pertaining to "Strain improvement of oyster mushrooms-*Pleurotus cystidiosus* O.K. Mill and *Pleurotus opuntiae* (Durieu and Lev.) Sacc." were undertaken in College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2018. Objective of the study was to standardize the techniques for production of *P. cystidiosus* and *P. opuntiae* and to study their morphological, physiological and cultural characteristics as well as nutritional and organoleptic qualities and to undertake genetic improvement by protoplast fusion. The methodologies used for these studies are described below.

3.1. SURVEY AND COLLECTION OF OYSTER MUSHROOMS

Random surveys were conducted during pre-monsoon and post-monsoon showers from November 2015 to July 2017 in different locations of Thiruvananthapuram district *viz.*, Vellayani and Chirayinkeeezhu of Kerala (Sumi, 2016). Specimens resembling the oyster mushrooms belonging to *P. cystidiosus* and *P. opuntiae* were collected at different stages of development and general observations pertaining to the natural occurrence and habitat of the spotted mushroom were identified and recorded. Places having dense vegetation of trees, forest ranges, meadows and fallen wooden logs were surveyed in each of the selected locations. Oyster mushrooms obtained from different surveyed locations were serially numbered and detailed laboratory studies were undertaken in the Department of Plant Pathology.

3.2. MORPHOLOGICAL STUDIES OF COLLECTED MUSHROOMS

Morphological studies of the collected oyster mushrooms (*P. cystidiosus* and *P. opuntiae*) were undertaken by detailed examination of macroscopic and microscopic characters of the sporocarps. The characters studied were recorded

following the techniques and data sheet developed by Nair (1990), as given in Appendix I.

3.2.1. Macroscopic studies

Macroscopic characters of the collected mushrooms *viz.,* colour, texture, nature of pileus, stipe, spore print, lamellae and gills were studied. Sporocarps closely resembling *P. cystidiosus* and *P. opuntiae* in their external features obtained during the survey were examined and sorted out for undertaking further studies.

3.2.1.1. Spore print

Spore print of the oyster mushrooms were obtained on black and dark pink paper sheets as per the procedure followed by Deepa (2016). A half matured sporocarp was selected for obtaining the spore print. The pileus was detached and placed on a piece of paper with gills facing the surface of the paper. A bell jar was placed over this, to keep the environment moist and to protect from air currents, in an air conditioned room at 28 ^{0}C for 10 h. Later, bell jar was removed and pileus was taken off from the paper to obtain the spore print.

3.2.2. Microscopic Studies

Microscopic studies of oyster mushrooms were conducted by adopting the standard technique (Deepa, 2016), using sections of fresh sporocarps of oyster mushrooms. Thin sections of the sporocarps were transferred to a glass slide, stained with lactophenol cotton blue (Appendix II) and examined under compound microscope (Carl Zeiss Primo star, Germany) (magnifications of 100X and 400X) for observing hyphal and mycelial characters of the mushrooms. The spores from the oyster mushrooms were measured directly after staining with

lactophenol cotton blue on a clean slide. Spores were thoroughly mixed with the stain to obtain a uniform spread and a cover slip was placed over it. Then, the average size of spores were measured under compound microscope (Carl Zeiss Primo star, Germany) (10X and 40X). Microphotographs of the spores were also taken to determine the shape and typical morphology of the fungal spore. Similarly, spores were observed after staining with 1 per cent congored. Hyphal characters *viz.,* colour of mycelium and nature of growth were studied by examining the actively growing mycelium of the mushroom culture obtained from tissue culture method.

3.3 ISOLATION AND PURE CULTURING

The pure culture of oyster mushrooms collected from the surveyed locations of Thiruvananthapuram were obtained by isolation adopting the standard tissue culture method (Suharban, 1987). Partially matured and healthy mushroom sporocarps collected during the surveys were cleaned thoroughly to remove any adhering soil particles, followed by surface sterilization with ethyl alcohol (99.9 per cent). The surface sterilized sporocarp was split longitudinally and a small portion of tissue from the junction of pileus and stipe was detached using a sterile inoculation needle. Detached tissue was transferred aseptically to petri dishes containing potato dextrose agar (PDA) medium and incubated for 7 to 10 days at $28 \pm 2\ ^{0}C$ to obtain pure growth of mushroom mycelium. The obtained cultures were again purified by hyphal tip method in which the hyphal tips of fungal growth were transferred aseptically to PDA slants (Rangaswamy and Mahadevan, 2008).

3.3.1. Maintenance of cultures

The purified fungal cultures of oyster mushroom isolates were sub cultured on PDA slants by cutting circular discs from the tip portion of mycelia, with the

help of a sterile cork borer of 5 mm size. The agar slants containing pure culture of the oyster mushrooms were maintained at 28 ± 1 ^{0}C for 15 days with subsequent revival of their growth at periodical intervals of 25 days. For the long term preservation of isolates, they were stored in a refrigerator at 5 ^{0}C and were revived at monthly intervals.

3.3.2. Selection of fast growing isolates

Seven isolates were obtained from survey *viz.*, two coremial (isolates from Vellayani) and two non-coremial isolates (isolates from Chirayinkeezhu and German) of *P. cystidiosus*; and three isolates of *P. opuntiae* (isolates from Vellayani). Isolates were screened for their rapid growth by recording the nature of mycelial growth and number of days taken for complete coverage of petri dishes (9 cm). Fast growing strains of *P. cystidiosus* (coremial and non-coremial) were separately selected, based on student t-test, with ten replications for each treatment (isolates). The experiment on isolates of *P. opuntiae* was laid out in Completely Randomised Design (CRD) with five replications for each isolate. Pure cultures of the fast growing isolates were sent to Directorate of Mushroom Research, Solan and accession numbers were retrieved.

3.4. MOLECULAR CHARACTERISATION OF OYSTER MUSHROOMS

Molecular characterisation and phylogenetic analysis of the fast growing isolates of *P. cystidiosus* (coremial and non-coremial) and *P. opuntiae* selected in 3.3.2. along with the two ruling varieties in Kerala *viz.*, *Pleurotus florida* (Mont.) Singer and *Pleurotus eous* (Berk.) were carried out *via* Internal Transcribed Spacer (ITS) sequencing.

3.4.1. Deoxy ribonucleic acid (DNA) isolation using NucleoSpin® Plant II kit

100 mg of fungal mycelium was homogenized using liquid nitrogen and powdered mycelium was transferred to a microcentrifuge tube. DNA isolation

was done using NucleoSpin® Plant II Kit (Macherey-Nagel, Germany), as per the manufacturer's guidelines and the eluted DNA was stored at 4 ^{0}C.

3.4.2. Agarose gel electrophoresis for DNA quality check

Quality of the isolated DNA was checked using agarose gel electrophoresis. 5 µl of the DNA sample was mixed with 1µl of 6X gel-loading buffer and was loaded to 0.8 per cent agarose gel prepared in 0.5X TAE buffer (Tris –Acetate- Ethylenediaminetetraacetic acid (EDTA) (TAE buffer) (pH 8.0) containing 0.5 µg ml^{-1} of ethidium bromide. Electrophoresis was performed with 0.5X TBE buffer at 75 V until, bromophenol dye front migrated to the bottom of the gel. The gel was then visualized and image was captured using BIORAD Molecular Imager (Gel DOC ^{TM} XR+) (Appendix III).

3.4.3. Polymerase chain reaction (PCR) Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (with 1.5 m*M* magnesium chloride), 0.2 m*M* each of deoxyribonucleotide triphosphates *i.e.* (dNTPs) *i.e.* dATP, dGTP, dCTP and dTTP, 1 µl of DNA, 0.2 µl of Phire Hotstart II DNA polymerase enzyme, 0.1 mg ml^{-1} of bovine serum albumin (BSA), 3 per cent dimethyl sulphoxide (DMSO), 0.5 M Betaine, along with 5 p*M* of forward and reverse primers.

3.4.3.1. Primers used

ITS refers to a piece of non-functional ribonucleic acid (RNA) situated between structural ribosomal RNAs on a common precursor transcript. PCR using ITS specific primers is used to determine the degree of variation between closely related species. ITS specific primers (White *et al.*, 1990) used in the current study were:

ITS Forward 5'- TCCGTAGGTGAACCTGCGG-3'

ITS Reverse 5'-TCCTCCGCTTATTGATATGC-3'

3.4.3.2. ***PCR amplification profile***

98 ^{0}C - 30 sec

98 ^{0}C - 5 sec
60 ^{0}C - 10 sec } 40 cycles
72 ^{0}C - 15 sec

72 ^{0}C - 60 sec

4 ^{0}C - ∞

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.4.4. Agarose Gel electrophoresis of PCR products

5 µl of PCR product was mixed with 1 µl of 6X loading dye and was loaded in 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide. Electrophoresis was performed with 0.5X TBE buffer at 75 V power supply for 1-2 h until bromophenol blue front has migrated to the bottom of gel. 2-log DNA ladder (New England BioLabs) was used as molecular standard. Gels were visualized and the image was captured in a BIORAD Molecular Imager (Gel DOC ^{TM} XR+).

3.4.5. ExoSAP-IT Treatment

ExoSAP-IT^{TM} PCR product cleanup reagent (GE Healthcare, UK) consisted of two hydrolytic enzymes *viz.*, Exonuclease I and Shrimp Alkaline Phosphatase (SAP) in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture. 5 µl of PCR product was mixed with 2 µl of ExoSAP-IT reagent and incubated at 37 ^{0}C for 15 min, followed by enzyme inactivation at 80 ^{0}C for 15 min.

3.4.6. Sequencing using BigDye Terminator v3.1

Sequencing of ITS region of oyster mushroom isolates, was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA) using

BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) following the manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP-IT treated)	- 10-20 ng
Primer	- 3.2 pM (Forward or Reverse)
Sequencing Mix	- 0.28 µl
Reaction buffer	- 1.86 µl
Sterile distilled water	- made up to 10 µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96 ^{0}C for 2 min followed by 30 cycles at 96 ^{0}C for 30 sec, 50 ^{0}C for 40 sec and 60 ^{0}C for 4 min.

3.4.7. Post Sequencing PCR Clean up

Master mix I comprised of 10µl milli Q and 2 µl 125 m*M* EDTA per reaction was prepared. Also, master mix II composed of 2 µl 3 *M* sodium acetate (pH 4.6) and 50 µl ethanol was prepared. Master mix I (12 µl) was mixed with master mix II (52 µl) and contents were mixed by inverting, followed by incubation at 26 ± 2 ^{0}C for 30 min. Mixture was further centrifuged at 14,000 rpm for 30 min and supernatant was decanted. Hundred µl of 70 per cent ethanol was added and spinned at 14,000 rpm for 20 min. The supernatant was decanted and pellet was air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems, USA).

3.4.8. Sequence Analysis

Quality of the sequence was checked using Sequence Scanner Software v1 (Applied Biosystems, USA). Alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

3.4.9. Characterisation

The morphological identity of five oyster mushroom isolates was confirmed by performing a similarity search using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database and the sequences were matched with existing NCBI database. The nucleotide sequences of five *Pleurotus spp.* (Appendix IV) were deposited in NCBI database and accession numbers were retrieved. Multiple nucleotide sequence alignment and phylogenetic analysis of the five *Pleurotus spp.* was done using Clustal-X software. Sequence phyllograms were constructed using Phylip package and unrooted trees were generated using TreeView software.

3.5. CULTURAL AND PHYSIOLOGICAL STUDIES

Cultural and physiological studies of the screened isolates of *P. cystidiosus* (coremial and non-coremial) and *P. opuntiae* were separately conducted by recording their growth on different media, temperature, pH, light conditions and with amendments.

3.5.1. Growth on different media

The growth characters of *P. cystidiosus* coremial, *P. cystidiosus* non-coremial and *P. opuntiae* were studied on four different solid media *viz.*, PDA, potato dextrose peptone agar (PDPA), oat meal agar (OMA), carrot extract agar (CEA) and malt extract agar (MEA). Three separate experiments were laid out in CRD with four replications for each treatment (media). Different media were prepared separately and autoclaved at 1.02 kg cm^{-2} pressure for 20 min and allowed to cool. Each of the sterilized media (20 ml) was poured into sterilized petri dishes and allowed to solidify. Mycelial disc of the selected isolate was inoculated on the medium, by cutting the mycelial mat from the periphery of 7 days old culture using a cork borer of diameter 5 mm and transferring it to the centre of the medium in the petri dish. The petri dishes were incubated at 28 ± 1 ^{0}C. Observations on radial colony growth was taken when the maximum mycelial

growth was attained in any one of the media tested. Other cultural characters *viz.*, colour, diameter and rate of mycelial growth were recorded when 50 per cent and 100 per cent growth was attained. The growth on each medium was recorded at 2 days interval and the best medium that supported maximum growth of each of the screened oyster mushroom isolate was used for further physiological studies. The data were statistically analysed and interpreted.The composition and procedures for preparation of the media used in the experiment were followed as described by Ainsworth *et al.* (1973). Chemical composition of the 5 media are given in Appendix V.

3.5.2. Growth at different temperature

Effect of temperature on the growth of *P. cystidiosus* coremial, *P. cystidiosus* non-coremial and *P. opuntiae* was studied at three different temperatures *viz.* 20 ^{0}C, 25 ^{0}C and 30 ^{0}C using the best basal medium that was screened in 3.5.1. Three separate experiments were laid out in CRD with four replications for each treatment (temperature levels). Sterilized, screened basal medium (20 ml) was poured into sterilized petri dishes. Each of the selected isolates was inoculated separately by transferring mycelial disc (5 mm diameter) from actively growing pure culture to the center of the plated medium followed by incubation at the temperatures specified above. Observations on nature of mycelial growth and time taken for complete growth on media at the different temperatures were recorded. The data were statistically analysed and interpreted.

3.5.3. Growth at different hydrogen ion concentration (pH)

The effects of different pH on growth characteristics of *P. cystidiosus* coremial, *P. cystidiosus* non-coremial and *P. opuntiae* were studied using the best basal medium that was screened in 3.5.1. Three separate experiments were laid out in CRD with four replications for each treatment (pH levels). The pH of the

screened medium was adjusted with pH meter using 0.1 *N* sodium hydroxide (NaOH) or hydrochloric acid (HCl). The pH of basal medium was adjusted to four levels *viz.,* 5.0, 6.0, 7.0 and 8.0. Then, the mycelial disc (5 mm diameter) was transferred from actively growing pure culture of the isolates separately and inoculated to the centre of the plated medium with different pH levels as specified above, followed by incubation at the temperatures most suited for the growth of isolates as observed in 3.5.2. Observations on nature of mycelial growth and time taken for complete growth of the different oyster mushroom isolates were recorded. The data were statistically analysed and interpreted.

3.5.4. Growth under light and dark conditions

The growth of *P. cystidiosus* coremial, *P. cystidiosus* non-coremial and *P. opuntiae* under light and dark conditions was studied using the best basal medium that was screened in 3.5.1. Three separate experiments were laid out in student t-test with 10 observations for each treatment. Treatments were light and dark conditions. The sterilized basal medium (20 ml) was poured into sterilized petri dishes, plated with best media having the optimum pH screened from 3.5.1. and 3.5.3. respectively. Each of the selected isolates was inoculated separately by transferring mycelial disc (5 mm diameter) from actively growing pure culture to the center of the plated medium. The plates were incubated at the best temperature (screened from 3.5.2.) in light and dark conditions. Light condition was facilitated by keeping the inoculated plates under tube light (1400 lux) whereas dark condition was facilitated by wrapping the inoculated plates with carbon sheets followed by incubation in closed cupboard. Observations on different fungal isolates *viz.,* nature of mycelial growth and time taken for complete coverage of petri dishes were recorded. The data were statistically analysed and interpreted.

3.5.5. Growth of *P. cystidiosus* coremial, *P. cystidiosus* non-coremial and *P. opuntiae* on medium added with different amendments

P. cystidiosus coremial, *P. cystidiosus* non-coremial and *P. opuntiae* were grown in the best basal medium, with best pH that was screened in 3.5.1 and 3.5.3 respectively, containing different amendments *viz.,* thiamine (25, 50 and 75 ppm), yeast extract (0.5, 0.75 and 1 per cent), iron (0.1, 0.125 and 0.15 per cent), gypsum *i.e.* calcium sulphate (2, 4 and 6 per cent) and calcium carbonate (2, 4 and 6 per cent). Three separate experiments were laid out in CRD with three replications for each treatment (amendments screened). The best basal medium with pH adjusted to the optimum level (screened for the different isolates) was separately prepared and sterilized by autoclaving at 15 lbs pressure for 20 min. The amendments *viz.*, yeast extract, iron (Ferrous sulphate), gypsum (calcium sulphate) and calcium carbonate was dissolved in distiiled water and added prior to sterilization, during the time of media preparation, whereas, thiamine (dissolved in distilled water and filter sterilized) was added to the sterilized and cooled media, followed by plating of amended media separately to petri dishes. After solidification of media, a culture disc (5 mm diameter), was transferred from 7 day old isolate and separately inoculated in different amended medium. Then incubation was done at the optimum temperature screened in 3.5.2. Observations on the nature of mycelial growth and time taken for complete coverage of petri dishes were recorded. The data were statistically analysed and interpreted.

3.6. SUBMERGED CULTURE PRODUCTION OF *P. CYSTIDIOSUS* COREMIAL, *P. CYSTIDIOSUS* NON-COREMIAL AND *P. OPUNTIAE*.

Submerged culturing of the screened oyster mushroom isolates *viz., P. cystidiosus* (coremial and non-coremial) and *P. opuntiae* were separately carried out to obtain pellets of fungal mycelium which has significance in mushroom processing industry. Liquid media *viz*., potato dextrose broth (PD broth), malt extract broth, carrot extract broth and potato dextrose peptone broth were prepared

and screened for the submerged culture production. Three separate experiments were laid out in CRD with four replications for each treatment (liquid broths). Composition and preparation of liquid broths were same as that of respective solid media, without the addition of solidifying agent, *viz*., agar-agar. Liquid media (100 ml) was taken in conical flasks (250 ml), followed by sterilization at 121 ^{0}C and 15 lbs pressure for 20 min. The sterilized liquid media were inoculated separately with mycelial discs (5 mm) obtained from the actively growing colony of the isolates. After 24 h of incubation, the conical flasks containing the inoculated liquid media were placed in a conical flask shaker (Amstrong Biotech Research, Bangalore) at 120 rpm (rotations per minute) for 30 days. Mycelial pellets formed from different mushroom isolates were collected by filtration using Whatman filter paper No. 1. Growth of fungal mycelium in liquid broths were recorded by taking observations on average size (mm), fresh weight (g L^{-1}), dry weight (g L^{-1}) and number of mycelial pellets formed per 100 ml of liquid medium. Size of mycelial pellets were recorded by taking the average size of 10 pellets. Dry weight of mycelial pellets were obtained by drying the filtered pellets in a mechanical drier (TEMCO, Instruments and Equipments, Chennai), until a constant weight was obtained. The data were statistically analysed and interpreted.

3.7. STUDIES ON THE COREMIAL CHARACTERISTICS AND ANAMORPHIC STAGE OF *P. CYSTIDIOSUS* (COREMIAL)

P. cystidiosus has specialized anamorphic structures called coremia on the culture assigned to a hyphomycete *viz*., *Antromycopsis macrocarpa* (Capelari, 1999). Coremial liquid of *P. cystidiosus* (coremial isolate) was mixed thoroughly with lacto phenol cotton blue on a clean slide to form a uniform spread and a cover slip was placed over it. Coremia is composed of conidia and average size of spores was measured using compound microscope (Carl Zeiss Primo star, Germany) (100X and 400X magnifications). Microphotographs were also taken to record the typical morphology and shape of the coremial spores.

3.7.1. Production, extraction and partial purification of extracellular melanin

Extracellular melanin pigment from the coremia of *P. cystidiosus* (coremial isolate) was extracted and partially purified by harsh acid-alkali method as described by Gadd (1982). Cultures of *P. cystidiosus* were maintained in petri dishes plated with PDA. Mycelial bits (5 mm diameter) of 6 days old *P. cystidiosus* cultures were inoculated to sterile PD broth (200 ml) in conical flask (250 ml) and incubated at 28 ± 2 ^{0}C under static condition for 35 days. A PDB without inoculation was kept as control. When the mycelium and broth changed to black in colour due to the accumulation of melanin pigment in the coremia, mycelial biomass was removed by filtration using whatman filter paper No. 1. The filtered culture fluid was adjusted to pH 10 by adding 1 *M* NaOH and the alkaline solution was autoclaved at 121 ^{0}C for 20 min. Again, the black alkaline solution was centrifuged at 5000 rpm for 5 min and supernatant was collected. Supernatant was acidified to pH 2 using concentrated 1 *N* HCl, followed by centrifugation at 5000 rpm for 5 min to precipitate the melanin. The precipitate was washed repeatedly with deionised water to remove the acid traces and dried at 4 ^{0}C for 10 h. The experiment was carried out in triplicate and the amount of pigment recovered was expressed as mg/l of culture broth.

3.7.2. Characterisation of the extracted melanin

3.7.2.1. Physical and chemical properties

The dried extracted melanin from *P. cystidiosus* coremial was characterised using physical and chemical tests proposed by Thomas (1955). Melanin nature of the pigment recovered from the culture filtrate of 35 days old *P. cystidiosus* was biochemically characterised by analysing (i) colour of the extracted solution in 1 *M* NaOH (ii) solubility in water, organic solvents (acetone, ethanol and hexane) and 1 *M* potassium hydroxide (KOH) (100 ^{0}C for 2 h), (iii) reaction with oxidizing agent *viz*., 3 per cent hydrogen peroxide (iv) precipitation in 3 *N* HCl (v) reaction to polyphenols (1 per cent ferric chloride) (vi) reaction with ammoniacal silver nitrate solution (Appendix VI).

3.7.2.2. Spectroscopic study by Ultraviolet (UV) spectrum

UV absorption spectra of the melanin was recorded as per the procedure followed by Tarangini and Mishra (2013). The extracellular melanin particles produced by fungus was dissolved in 3 ml of borate buffer (pH 8.0) (Appendix VI) and its UV spectrum was recorded from 200-1000 nm with UV/Visible Spectrophotometer (Beckman Coulter DU-640, USA).

3.7.3. Antioxidant activity of the extracellular melanin

Antioxidant activity of melanin was recorded by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (William *et al.*, 1995). Antioxidant compounds will reduce DPPH and change its colour from deep violet to light yellow. Intensity of yellow colour was measured at 515 nm with UV/Visible spectrophotometer (Beckman Coulter DU-640, USA). Extracted melanin was dissolved in 95 per cent ethanol and different concentrations *viz.*, 200, 400, 600 and 800 µg/ml were prepared. 0.1 mM DPPH solution (1 ml) prepared in 95 per cent ethanol was added to the extracted melanin prepared at different concentrations. The different mixtures were incubated in dark at 40 ^{0}C for 30 min and absorbance was measured at 516 nm against a blank. Ascorbic acid was used as reference standard. Control was prepared without the addition of melanin. 95 per cent methanol was used as blank. The inhibitory concentration (IC 50) was the concentration of sample required to inhibit 50 per cent of the DPPH free radicals and was calculated using log dose inhibition curve. Lower absorbance of the reaction mixture indicated higher free radical activity. The experiment was carried out in triplicate and per cent DPPH scavenging effect or per cent inhibition was calculated by the equation:

$$: \frac{\text{Absorbance of control (0 minute)-Absorbance of test sample x100}}{\text{Absorbance of control at 15 minutes}}$$

3.8. EVALUATION OF DIFFERENT SUBSTRATES FOR SPAWN PRODUCTION OF *P. CYSTIDIOSUS* COREMIAL, *P. CYSTIDIOSUS* NON-COREMIAL AND *P. OPUNTIAE*

Different substrates *viz.*, grains (paddy grains, sorghum) and rubber sawdust were evaluated for the spawn production of the mushrooms *viz.*, *P. cystidiosus* (coremial and non-coremial) and *P. opuntiae*. The procedure for the spawn production was followed according to the standard techniques described below.

3.8.1. Grain spawn production (Sinden, 1934)

The grains of paddy and sorghum were cooked in boiling water until seed coat had just begun to split open. Precaution should be taken to avoid full opening of grains as these would enhance the growth of other microbial contaminants. The excess water was then drained off and grains were spread out for drying, on a clean cloth laid out on the floor. After sufficient drying, each of the grain substrate was mixed with calcium carbonate (40 g per kg of grains). This mixture was packed separately in polypropylene bags of size 12 inches x 6 inches (300 g of the mixture per bag) and sterilized by autoclaving at 121 ^{0}C, under 1.02 kg cm^{-2} pressure, for 2 h. After cooling, the bags were inoculated aseptically, with mycelial bits from 10 days old culture of the screened isolates separately and incubated at 28 $\pm$ 2 ^{0}C for complete mycelial coverage of the spawn substrate. The spawn thus obtained served as mother spawn for further spawn production and amendment studies.

3.8.2. Sawdust spawn production (Deepa, 2016)

After removing the clods and other impurities rubber sawdust was soaked overnight, in water for 12 h. Then the excess water was drained off and sawdust was allowed to dry. The sawdust was then mixed thoroughly with calcium carbonate (40 g per kg of grains) along with simultaneous sprinkling of water to

attain 60 per cent moisture. The sawdust-calcium carbonate mixture was then filled in polypropylene bags and sterilized at 121 ^{0}C for 2 h with 1.02 kg cm^{-2} pressure in an autoclave. Mycelial bits from 10 days old pure culture of oyster mushroom isolates were separately inoculated into the cooled sawdust-calcium carbonate mixture under aseptic conditions and incubated at 28 $\pm$ 2 ^{0}C for complete spawn run in the substrate. The spawn thus obtained as mother spawn was stored for further spawn production and amendment studies.

3.8.3. Evaluation of different amendments for spawn production

Enrichment of spawn substrates with various amendments significantly accelerated the mycelial spread and increased the biological efficiency. In this context, spawns prepared from the substrates *viz.*, paddy grains, sorghum and rubber sawdust were separately amended with additives at different concentrations *viz.*, yeast extract (0.5, 0.75 and 1 per cent), iron in the form of ferrous sulphate (0.25, 0.5 and 0.75 per cent) and thiamine (25, 50 and 75 ppm). Nine experiments (for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae*) with each substrate and respective nine amendments were laid out separately in CRD, to find the best amendment in each substrate with three replications for each treatment. Treatments were yeast extract (0.5, 0.75 and 1 per cent), iron in the form of ferrous sulphate (0.25, 0.5 and 0.75 per cent) and thiamine (25, 50 and 75 ppm). Also, three experiments (for the three oyster mushroom isolates) with three substrates and respective nine amendments were laid out separately in CRD to find the best spawn substrate with the best amendment with thirty treatments and three replications for each treatment. Spawns prepared from the substrates alone (without amendments) served as controls. Substrate-calcium carbonate mixture was prepared using grains and rubber sawdust separately as per the standard procedure described in 3.8.1. and 3.8.2. The amendments were separately dissolved in warm distilled water and mixed with the prepared mixtures to attain the final moisture content of 60 per cent. The amended sawdust-calcium carbonate mixture was then filled in

polypropylene bags, followed by sterilization, inoculation and incubation as per the standard procedures. The best spawn substrate with the best amendment was selected for further studies based on following parameters. The data were statistically analysed and interpreted.

3.8.4. Parameters deciding the efficacy of different substrates and amendments on spawn production

In order to determine the efficacy of different substrates and amendments on spawn production, three main characters were taken into consideration *viz.*, time taken for spawn run, nature of mycelial growth and presence of contaminants.

3.8.4.1. Time taken for spawn run (days)

The spawn run period (the number of days from inoculation to complete colonization of substrate by the mycelium) was recorded.

3.8.4.2. Nature of mycelial growth

After the completion of spawn run, observations *viz.*, growth pattern of mycelia, change in colour and nature of spawn run were recorded, in each of the above amended substrates and control.

3.8.4.3. Presence of contaminants

Amended spawns and controls were periodically observed. Percentage of fungal and bacterial contamination in each treatment was recorded.

3.8.4.4. Shelf life (days)

Number of days up to which amended spawns and control spawn can be stored without deterioration were recorded.

Following scale was used for assessing the myelial growth pattern of spawn (Priya *et al*., 2017):

++++ : Thicker and fluffy growth

+++ : Thick growth

++ : Poor growth

Good quality mother spawns were produced using the substrates and amendments screened on the basis of parameters mentioned above. Grower's spawns were produced from the good quality mother spawn and were further used for the preparation of the mushroom beds.

3.9. CULTIVATION TRIALS OF *P. CYSTIDIOSUS* COREMIAL, *P. CYSTIDIOSUS* NON-COREMIAL AND *P. OPUNTIAE*

Standardization of the substrate for efficient production of the oyster mushroom spawns was followed by evaluating the locally available substrates *viz.*, paddy straw, sawdust of rubber tree and neopeat for the production of oyster mushroom isolates *viz., P. cystidiosus* (coremial and non-coremial) and *P. opuntiae* according to the procedure given by Baskaran *et al*. (1978).

3.9.1. Polybag method using paddy straw as substrate

Chemical sterilization of paddy straw was carried out by soaking paddy straw in water containing 75 ppm carbendazim (bavistin) and 500 ppm formalin for 18 h. The excess water was drained off and straw was spread over a silpaulin sheet under sun. Straw was dried under sun until the moisture content was reduced to 60 per cent. The mushroom beds were prepared using polypropylene covers of 60 cm x 30 cm size (thickness of 100 mm gauge), following the polybag method of cultivation. Solar dried paddy straw was placed in the polybag as twists and

spawn was laid over the twists towards the sides. Over the spawn layer, again paddy straw twist was laid followed by spawning. Likewise, four layers were prepared and the upper paddy straw layer was completely covered with spawn. The bags were made compact, tied firmly at the top with a nylon strip and provided with 15 pin holes for sufficient air circulation. For each bed, 1000 g (dry weight) of paddy straw and 150 g of spawn were used.

3.9.2. Polybag method using saw dust as substrate

Saw dust of rubber tree was soaked in water for 24 h and excess water was drained off the next day. The drained material was sun dried, mixed with 3 per cent calcium carbonate and filled in polypropylene covers (60 cm x 30 cm size, with thickness of 100 mm gauge). The mixture was sterilized at 121 ^{0}C for 2 h under 1.02 kg cm^{-2} pressure in an autoclave. Mushroom beds were prepared using the sterilized mixture following the procedure described in 3.9.1. For each bed, 1000 g (dry weight) of sawdust and 150 g of spawn were used.

3.9.3. Polybag method using neopeat as substrate

Neopeat blocks were soaked in water for 24 h. Excess water was drained off the next day followed by steam sterilization in order to prepare the mushroom beds as in 3.9.1. 2. For each bed, 1000 g (dry weight) of neopeat and 150 g of spawn were used.

3.9.4. Evaluation of different amendments for polybag method of mushroom production

Agricultural wastes used for the preparation of mushroom beds are very poor in nitrogen content and therefore deliberative supplementation of substrates with different nitrogen rich compounds significantly increase the mushroom yield and nitrogen content in sporophores. In this background, nitrogen supplements *viz.,* wheat bran (2 and 4 per cent), rice bran (2 and 4 per cent), neem cake (2 and 4 per cent) and chemical sprays *viz.*, urea (0.5 and 1 per cent), gypsum (1 and 2 per cent), 1 *M* potassium dihydrogen phosphate (KH_2PO_4) (2 and 2.5 per cent)

and gibberellic acid (GA) (10 and 20 ppm) were used as amendments for the different substrates *viz.*, paddy straw, rubber wood sawdust and coirpith in polybag method of cultivation. Nine experiments (for *P. cystidiosus* (coremial and non-coremial) and *P. opuntiae*), with each bed substrate and respective fourteen amendments were laid out separately in CRD, to find the best amendment in each bed substrate with three replications for each treatment. Three separate experiments (for the three oyster mushroom isolates), with three bed substrates and respective fourteen amendments were laid out separately in CRD to find the best bed substrate with the best amendment with fourty five treatments and three replications for each treatment. Here, beds prepared from the substrates alone (without amendments) served as the controls.

Each of the bed substrate was processed according to the standard procedure and mixed separately with the nitrogen supplements (20 g and 40 g per kg of substrate). The mixture was filled in polypropylene cover (60 cm x 30 cm size, with thickness of 100 mm gauge) and steam sterilized for 1 h. Mushroom beds were prepared in polypropylene covers of 60 x 30 cm size (thickness of 100 mm gauge) using the sterilized amended substrate. Spawning was done layer by layer up to four layers by filling the polythene bag with spawn and amended substrates. The prepared polybags were made compact by tying at the top and few holes were provided for air circulation. The beds were then transferred to incubation chamber for initiation of spawn run. After the completion of spawn run, beds were transferred to cropping room for fruiting and one-inch slits (8-10 in number) were made in the polybags for the emergence of pinheads. Chemicals *viz.*, urea (0.5 and 1 per cent), gypsum (1 and 2 per cent) and 1 *M* KH_2PO_4 (2 and 2.5 per cent) were dissolved in water to prepare the required concentrations. Stock solution of GA (50 ppm) was prepared in 95 per cent ethanol and was used for the preparation of working standards *viz.*, 10 and 20 ppm. After completion of spawn run, the spawn bag was opened at the top and one inch slits (8-10 in number) were made around the beds, for spraying the required chemical solutions at three stages

of mycelial run (500 ml each) *viz.*, at complete spawn run, after first harvest and 15 days later. Oyster mushrooms were picked when young and preferably in clusters. The best bed substrate and amendment was selected for further studies based on following parameters.

3.9.5. Parameters deciding the efficacy of different substrates and amendments on mushroom production

Best substrate and amendment for each oyster mushroom isolate was selected based on the parameters *viz.*, number of days taken for the development of mushroom sporocarps (from pinhead stage to maturity stage), total yield per bed from three harvests, total crop period, average weight of sporocarp, number of sporocarps, biological efficiency, along with the incidence of microbial contaminants and insect pests.

3.9.5.1. Developmental morphology

Developmental morphology of each screened isolate was studied by recording and observing the developmental changes of sporocarps from pinhead stage to harvesting stage. Observations on the colour, size and shape of the sporocarps were recorded. Also, the time taken for development of pinheads to mature (harvest) stage was recorded.

3.9.5.1.1. Time taken for complete spawn run (days)

Number of days required for the complete colonization of the bed substrates by the fungal mycelium upon inoculation was recorded.

3.9.5.1.2. Time taken for pinhead formation (days)

Number of days taken for the initiation of pinheads after the completion of mycelial growth and number of pinheads formed were recorded.

3.9.5.1.3. Time taken for first harvest (days)

Number of days taken by the pinheads to complete their growth and expansion was recorded.

3.9.5.2. Total yield per bed from three harvests (kg bed^{-1})

Total yield per bed was calculated as the ratio of total weight of all the mushrooms harvested from three harvests to the dry weight of substrate used for cultivation.

3.9.5.3. Total crop period (days)

Total number of days taken from the time of spawn inoculation till the last harvest of sporocarps from the beds was recorded.

3.9.5.4. Average weight of sporocarp (g)

Average weight of sporocarp was obtained by dividing the total weight of mushrooms harvested by total number of mushrooms harvested.

3.9.5.5. Number of sporocarps

Total number of mushrooms picked up to three harvests was recorded.

3.9.5.6. Biological efficiency (BE) (per cent)

$$\text{B.E (per cent)} = \frac{\text{Total weight of fresh mushrooms harvested per polybag (g)}}{\text{Dry weight of substrate per polybag (g)}} \times 100$$

3.9.5.7. Incidence of microbial contaminants and insect pests

Incidence of pest and diseases were observed during the time of spawn run as well as mushroom production. The pests and diseases were identified in the respective laboratories at the College of Agriculture, Vellayani and per cent incidence was recorded. Simultaneously, the contaminated beds were removed as and when observations were made. The best substrate and amendment were

selected based on the parameters discussed above and used for the further comparative studies. The data were statistically analysed and interpreted.

3.10. COMPARATIVE EVALUATION OF THE PERFORMANCE OF *CYSTIDIOSUS* COREMIAL, *P. CYSTIDIOSUS* NON-COREMIAL AND *P. OPUNTIAE* WITH COMMON OYSTER MUSHROOMS (*P. FLORIDA* AND *P. EOUS*).

A comparative evaluation on the performance of the screened isolates of *P. cystidiosus* (non-coremial) and *P. opuntiae* with *P. florida* and *P. eous* respectively was made using the screened spawn medium and cultivation substrate amended with the respective additives. Spawns of the *Pleurotus spp.* isolates were prepared using the best amended spawn medium screened in 3.8.4 as per the standard procedure and mushroom beds were prepared with the amended substrate screened in 3.9.4. using polybag method. Observations on the time taken for spawn run and nature of mycelial growth were recorded in case of spawn production for all the evaluated mushroom isolates. The developmental morphology, total yield, total crop period, average weight of sporocarp, number of sporocarps and B.E were recorded.

Comparative spawn production experiment with *P. cystidiosus* and *P. florida* was laid out in CRD with 4 replications for each treatment. Five treatments were spawns of *P. cystidiosus* (coremial) and *P. florida* prepared on sorghum grains amended with yeast 1 per cent; and spawns of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. florida* prepared on paddy grains amended with yeast 1 per cent. Comparative spawn production experiment with *P. opuntiae* and *P. eous* was laid out in CRD, with 4 replications for each treatment. Four treatments were spawns of *P. opuntiae* and *P. eous* prepared on sorghum grains amended with yeast 1 per cent; and paddy grains amended with yeast 1 per cent. Comparative cultivation experiment with *P. cystidiosus* (non-coremial) and *P. florida* was laid out in student t-test with 10 observations for each treatment. Two

treatments were *P. cystidiosus* (non-coremial) and *P. florida* on rubber wood sawdust sprayed with 2.5 per cent of 1 *M* KH_2PO_4. Comparative cultivation experiment with *P. opuntiae* and *P. eous* was laid out in CRD, with 5 replications for each treatment. Four treatments were *P. opuntiae* and *P. eous* on rubber wood sawdust separately amended with wheat bran (4 per cent) and neem cake (4 per cent). The data obtained were compared and interpreted. Also, beds of *P. cystidiosus* (non-coremial) and *P. opuntiae* were laid out in three seasons *i.e.*, February to May, June to September and October to January of 2016-17 to find out the effect of different seasons on mushroom production. The weather data in three seasons of 2016-17 is given in Appendix VII. Trials with *P. cystidiosus* (non-coremial) and *P. opuntiae* were carried out at Vellayani, Thiruvananthapuram and Thankamany, Idukki to study their comparative performance in different locations. Six experiments were separately, laid out in student t-test with 10 observations for each treatment. The four treatments were *P. opuntiae* in Vellayani and Idukki; and *P. cystidiosus* (non-coremial) in Vellayani and Idukki. The data were statistically analysed and interpreted.

3.11. CROPPING CONDITIONS

Cropping conditions for fruiting phase are quite different from that of spawn run phase. The inoculated substrates were kept in a spawn running room at 25 ^{0}C and 85-90 per cent relative humidity under dark conditions. After the complete spawn run, polybags were moved to a cropping room, at 28 ^{0}C, 80 per cent or above relative humidity and light intensity of 100 lux. The walls and floor of cropping room was sprayed with water twice a day to maintain humidity during the cropping time.

3.12. ANALYSIS OF PROXIMATE CONSTITUENTS

Proximate constituents of *P. cystidiosus* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous viz.*, moisture content, carbohydrate, reducing sugar, polysaccharides *viz.*, cellulose and starch, protein, fat, fibre, ash, total

antioxidants, beta-carotene, polyphenols and energy were estimated and compared. Dried and powdered oyster mushroom fruiting bodies were used for the different analysis, except for the estimation of moisture content. Thirteen experiments were laid out separately in CRD for each proximate constituent, with four replications and four treatments (mushrooms). The data were statistically analysed and interpreted.

3.12.1. Estimation of moisture content

Fresh samples of the oyster mushroom isolate was weighed (w_1) and dried in a hot air oven until a constant weight was obtained (w_2). The difference between fresh and dried weight of the sample was expressed as percentage (Geetha, 1993).

$$\text{Per cent moisture content (\%)} = \frac{w_1 - w_2}{w_1} \times 100$$

3.12.2. Estimation of carbohydrate

Total carbohydrate content of *Pleurotus spp*. was estimated using anthrone method (Aminoff *et al*., 1970). Dried and powdered mushroom sample (100 mg) was weighed out and hydrolysed with 5ml of 2.5 *N* HCl, in a boiling water bath for 3h. The hydrolysate was then cooled at 26 ± 2 ^{0}C and neutralised with solid sodium carbonate until the effervescence ceased. The volume was made to 100 ml and centrifuged at 5000 rpm for 10 min. From the supernatant, 0.5 ml of aliquot was taken and made up to 1 ml by adding distilled water. To this, 4 ml of anthrone reagent was added and heated for 8 min in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118). Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.

3.12.3. Estimation of reducing sugars

3.12.3.1. Extraction of reducing sugars

Reducing sugars was extracted from the sample using Nelson-somogyi's method (Somogyi, 1952). Mushroom sample (100 mg) was extracted twice with hot 80 per cent ethanol (5ml each time). Supernatant was collected and evaporated on a boiling water bath at 80 ^{0}C, followed by the addition of water (10 ml) to dissolve the extracted reducing sugars.

3.12.3.2. Estimation of reducing sugar

Estimation of sugar was done by dinitrosalicylic acid method (Miller, 1972). When alkaline solution of 3,5-dinitrosalicylic acid (DNSA) reacts with reducing sugars (*viz.*, glucose, galactose, lactose and maltose), it gets converted in to orange coloured 3-amino-5-nitrosalicylic acid. 0.5 ml of ethanol-free extract obtained from 3.12.2.1.1. was pippeted into test tube and made up to 3 ml with water. 3 ml of DNSA reagent was then added and heated for 5 min in a boiling water bath. Dinitrosalicylic acid (DNSA) reagent was prepared by dissolving 1g of dinitrosalicylic acid, 200 mg of crystalline phenol and 50 mg of sodium sulphite in 100 ml of 1 per cent NaOH solution. Yellowish orange reagent was stored in a stoppered bottle at 4 ^{0}C. After the development of orange reddish colour, 1ml of 40 per cent warm Rochelle salt (Potassium sodium tartrate tetrahydrate) solution was added, mixed and cooled under running tap water. Absorbance was measured at 510 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118), using reagent blank adjusted to zero absorbance. The amount of reducing sugar in the sample was calculated using a standard graph prepared from working standard glucose solutions (0 to 500 µg) in the same manner.

3.12.4. Estimation of polysaccharide

Estimation of polysaccharides included the estimation of cellulose and starch.

3.12.4.1. Estimation of cellulose

Cellulose was estimated as per the standard procedure followed by Updegroff (1969). Cellulose undergoes acetolysis with acetic/nitric reagent to form acetylated cellodextrins which get dissolved and hydrolysed to form glucose units on treatment with 67 per cent sulphuric acid (H_2SO_4). On dehydration, glucose forms 5-hydroxymethyl furfural which forms a green coloured product with anthrone reagent and the colour intensity is measured at 630 nm. 3 ml of acetic: nitric reagent (150 ml of 80 per cent acetic acid and 15 ml of concentrated nitric acid) was added to 1 g of the sample, mixed using a vortex mixer and placed in a boiling water bath for 3 min. The mixture was cooled and centrifuged at 6500 rpm for 15-20 min. Supernatant was discarded and the residue was washed with distilled water. To the residue, 10 ml of 67 per cent H_2SO_4 was added and left for 1 h. 1 ml of this solution was then diluted to 100 ml. 10 ml of anthrone reagent was added to 1 ml of the diluted solution and heated in a boiling water bath for 10 min. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118). Blank was prepared using anthrone reagent and water. Cellulose solution was used as standard. Standard cellulose solution was prepared by adding 100 mg of cellulose in 10 ml of 67 per cent sulphuric acid and 1 ml of this solution diluted to 100ml (100µg/ ml). Cellulose in the sample was calculated from the standard graph and expressed as mg/g.

3.12.4.2. Estimation of starch

Estimation of starch was done using anthrone reagent (Hedge and Hofreiter, 1962). The sample was treated with 80 per cent alcohol to remove sugars and then starch was extracted with perchloric acid. In the hot acid medium, starch was hydrolyzed and dehydrated to form glucose and hydroxymethyl furfural respectively. Further, hydroxymethyl furfural formed a green coloured product on reaction with anthrone reagent. 0.5 g of dried mushroom sample was homogenised in hot 80 per cent ethanol, followed by centrifugation at 2500 rpm for 5 min. The retained residue was repeatedly washed with hot 80 per cent

ethanol till the the washings became colourless. The washed residue was dried over a boiling water bath for 3 h. 5 ml of distilled water and 6.5 ml of 52 per cent perchloric acid was added to the dried residue and extraction was done at 0 ^{0}C for 20 min, followed by centrifugation at 2500 rpm for 5 min. Supernatant was saved and extraction followed by centrifugation was again repeated. 0.2 ml of the saved supernatant was pipetted out and made up to the 1 ml with distilled water. 4 ml of anthrone reagent was added to the contents and were heated for 8 min in a boiling waterbath, followed by rapid cooling. The intensity of green to dark green colour was read at 630 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118). Standard starch solution was prepared by taking 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard in test tubes and made up to 1ml with distilled water. Glucose content in the sample was estimated using the standard graph. Multiplication of the graph value by a factor 0.9 gave the estimated starch content of the sample.

3.12.5. Estimation of total soluble protein

Protein content of *Pleurotus spp.* was estimated by Lowry's method (Lowry *et al.,* 1951), using Folin-Ciocalteau reagent (FC Reagent). Components of FC *viz.*, phospho molybdic acid and phospho tungstic acid are reduced by aminoacids *viz.,* tyrosine and tryptophan present in the sample.

3.12.5.1. Extraction of total soluble protein

0.5 g of dried mushroom sample was ground in mortar with potassium phosphate buffer (Appendix VIII) and centrifuged at 5000 rpm for 15 min at 4 ^{0}C. The supernatant was saved for the estimation of soluble protein. Samples with high phenolic or pigment content, were extracted with a reducing agent *viz.,* cysteine or sodium chloride, followed by precipitation and separation of protein with trichloroacetic acid.

3.12.5.2. Estimation of total soluble protein

Alkaline copper solution was prepared by mixing 50 ml of 20 per cent sodium carbonate in 0.1 *N* NaOH with 1 ml of 0.5 per cent copper sulphate in 1 per cent potassium sodium tartrate solution. 0.2 ml of the sample extract was pipetted into test tube and made up to 1 ml, with distilled water. 5 ml of the alkaline copper solution was added to the sample extract, mixed well and incubated at 27 ± 2 ^{0}C for 10 min. After incubation, 0.5 ml of FC reagent was added and incubated at 27 ± 2 ^{0}C in dark for 30 min. After incubation, absorbance of blue colour was read at 660 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118) against reagent blank. BSA was used as the protein standard, by pipetting out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution into series of test tubes and was made up to 1 ml. Protein content in the sample was calculated from standard graph and expressed as mg/g of the sample.

3.12.6. Estimation of fat

Estimation of fat in the screened isolates was carried out as per the procedure of Lees (1975). Dried mushroom powder (5g) was taken in a thimble and kept inside the Soxhlet extraction apparatus. Extraction of sample was then carried out using petroleum ether for 16 h. A piece of cotton wool was placed at the top of thimble during the extraction for the proper distribution of solvent in the sample. The extract was transferred into a pre-weighed beaker (w_1), cooled in a desiccator and again weighed (w_2). The percentage of fat in the sample was then determined using the following equation.

$$\text{Percent fat content (\%)} = \frac{w_2 - w_1}{\text{weight of the sample}} \times 100$$

3.12.7. Estimation of crude fiber

Crude fiber content of the screened isolates was estimated as per the procedure described by De (1965). Powdered mushroom sample (2 g) was extracted with petroleum ether to remove the fat content. After extraction, the

sample was boiled with 200 ml of concentrated sulphuric acid (1.25 per cent) for 30 min, with bumping chips. The digested sample was then filtered through a muslin cloth and washed with boiling water until washings are no longer acidic. The sample was again boiled with 200 ml of NaOH for 30 min and filtered through muslin cloth. Again the sample was washed with 25 ml of boiling 1.25 per cent sulphuric acid, followed by 50 ml of water and 25 ml of alcohol. The sample was transferred to a pre-weighed silica crucible (w_1) and dried for 2 h at 130 $\pm$ 2 ^{0}C. Dish was cooled in a desiccator and weighed (w_2). The residue was further ignited at 600 $\pm$ 15 ^{0}C for 30 min, cooled in a desiccator and reweighed (w_3).

$$\text{Per cent crude fiber (\%)} = \frac{\text{loss in weight on ignition } i.e.\ (w_2\text{-}w_1)\text{-}(w_3\text{-}w_1)}{\text{weight of the sample}} \times 100$$

3.12.8. Estimation of ash

Dried mushroom powder (5 g) was transferred to a pre-weighed silica crucible (w_1). It was then heated at low flame over a Bunsen burner and the charred substrate was transferred to a muffle furnace. It was then heated at 500 ^{0}C for 2 h, until a white ash was obtained. Weight of the ash (w_2) was recorded after cooling in a desiccator. Per cent ash content was estimated using the following formula (Raghuramulu *et al.*, 1983).

$$\text{Per cent ash content} = \frac{\text{loss in weight } i.e.\ w_2\text{-}w_1}{\text{weight of sample}} \times 100$$

3.12.9. Estimation of total antioxidants

Total antioxidants of the mushroom isolates were estimated by the phospho molybdenum method, as per the procedure described by Prieto *et al.* (1999). Dried mushroom powder was extracted in ethanol and 0.3 ml of the

extract was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 m*M* sodium phosphate and 4 m*M* ammonium molybdate). Test tubes containing the reaction mixture were incubated at 95 ^{0}C for 90 min. Then, the solution was cooled at 27 ± 2 ^{0}C and absorbance was measured at 695 nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118) against blank. Methanol (0.3 ml) was used as blank and the total antioxidant content was expressed as µg per equivalent g of ascorbic acid.

3.12.10. Estimation of beta carotene

Estimation of beta carotene of mushroom samples was carried out by the standard procedure given by Srivastava and Kumar (1994). Dried mushroom powder (5 g) was crushed in mortar with 10-15 ml of acetone and a few crystals of anhydrous sodium sulphate. The supernatant obtained was decanted into a beaker and the process was repeated twice. The supernatants were combined, transferred to a separating funnel and mixed thoroughly with 10-15 ml of petroleum ether. When the two layers got separated out in the separating funnel lower layer was discarded. The upper layer was collected in a 100 ml volumetric flask and the volume was made to 100 ml with petroleum ether. Optical density (O.D.) was measured at 452 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118) and petroleum ether was used as blank.

$$\beta\text{- carotene content } (\mu g/100g) = \frac{\text{O.D. x } 13.9 \text{ x } 10^4 \text{ x } 100}{\text{Weight of sample x } 560 \text{ x } 1000}$$

3.12.11. Estimation of polyphenols

Total amount of polyphenols in the mushroom samples was measured by Folin-Ciocalteu method (Ondo *et al.*, 2013). Dried mushroom sample (10 g) was macerated and extracted with 150 ml of 50 per cent ethanol, for 72 h. The extract was filtered and dried at 40 ^{0}C, under reduced pressure. Dried extract was mixed

with 1.25 ml of FC reagent (0.2 *N* diluted in methanol) and incubated at 26 ± 2 ^{0}C, for 5 min. After incubation, 1 ml of 1.5 *N* sodium carbonate solution was added and sample was again incubated at 26 ± 2 ^{0}C for 2 h. The absorbance was measured at 765 nm and methanol was used as reagent blank. Total polyphenol content was expressed as mg of gallic acid equivalents (GAE) per g of sample extract.

3.12.12. Estimation of energy

The energy value of *Pleurotus spp.* was estimated, based on the contents of protein, fat and carbohydrate, in the respective mushroom samples, using the factors 2.62, 8.37 and 4.2 (Crisan and Sands, 1978). Energy value (Kcal per 100 g of dry weight) = 2.62 x (per cent protein content) + 8.37 x (per cent fat content) + 4.2 x (per cent carbohydrate content)

3.13. DETERMINATION OF ANTI-CANCEROUS ACTIVITY

The MTT (Microculture Tetrazolium Assay) or in vitro cell proliferation assay is one of the most widely used assay, for evaluating preliminary anticancer activity of synthetic derivatives and natural products (Cauley *et al.*, 2013). In the present study, invitro anti-proliferative activity of *P. cystidiosus* (non-coremial) and *P. opuntiae* isolates against colon cancer cell line (HeLa) was determined. Here, per cent viability of cancer cell line with different concentrations of *Pleurotus* spp. extracts and their respective IC 50 values (µg ml^{-1}) were compared. Two separate experiments were laid out in student t-test with 10 replications for each treatment (mushroom).

The cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecos modified eagles medium (DMEM) (Gibco, Invitrogen). The cell line was cultured in tissue culture flask (250 ml) containing DMEM supplemented with 10 per cent fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution (penicillin (100

μg ml^{-1}), streptomycin (100 μg ml^{-1}), and amphotericin B (2.5 μg ml^{-1}). Cultured cell lines were kept at 37 ^{0}C in a humidified, 5 per cent carbon dioxide (CO_2) incubator (Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera), followed by MTT assay as per the procedure described by Talarico *et al.*, (2005).

3.13.1. Seeding of cancer cell lines in 96 well plate

Two days old confluent monolayer of cervical cancer cells was trypsinized and the cells were suspended in 10 per cent DMEM. 100μl of the cell suspension (5 x 10^4 cells $well^{-1}$) was seeded in 96 well tissue culture plate and incubated at 37 ^{0}C for 24 h in a humidified, 5 per cent CO_2 incubator.

3.13.2. Preparation of mushroom extract

Dried and powdered mushroom sporocarp extracted in ethanol (1ml) was mixed with 1ml of DMEM and dissolved completely by cyclomixer. The extract mixture was filtered through millipore syringe filter (0.22 μm) to ensure its sterility.

3.13.3. Evaluation of antiproliferative activity

Freshly prepared mushroom sample in 5 per cent DMEM was serially diluted to concentrations *viz.*, 100, 50, 25, 12.5 and 6.25 μg in 100 μl of 5 per cent DMEM. Each concentration (100 μl) was added in triplicate to the respective wells (plated with trypsinized and suspended cervical cancer cells) and incubated at 37 ^{0}C in a humidified, 5 per cent CO_2 incubator.

3.13.4. Antiproliferative assay by direct microscopic observation

The entire tissue culture plate was observed with inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera), at intervals of 24 h; up to 72 h and microscopic observations were recorded as images. Detectable changes in the morphology of the cells *viz.,* rounding or

shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

3.13.5. Antiproliferative assay by MTT Method

15 mg of MTT (Sigma, M-5655) was dissolved in 3 ml of phosphate buffer saline, pH 7.4 (PBS) (Appendix IX) and sterilized by filter sterilization. 30 µl of reconstituted MTT solution was added to the test and control wells in **3.13.3**. The plate was gently shaken and incubated at 37 ^{0}C in a humidified, 5 per cent CO_2 incubator for 4 h. The supernatant was removed, 100 µl of MTT solubilization solution (DMSO) was added to the wells and mixed gently by pipetting, in order to solubilize the formazan crystals. The absorbance values of sample and control wells, were measured at 570 nm using microplate reader (Microplate Reader 680, BIORAD). The percentage of viability of cancer cell line was calculated using the following formula.

$$\text{: } \frac{\text{Absorbance value of control-Absorbance value of sample}}{\text{Absorbance value of control}} \times 100$$

The inhibitory concentration (IC 50) is the concentration of mushroom sample required to inhibit 50 per cent of the DPPH free radicals. IC 50 value was calculated using log dose inhibition curve.

3.14. KEEPING QUALITY

Keeping quality of *P. opuntiae* and *P. cystidiosus* (non-coremial) isolates were evaluated, using sporocarps of medium maturity. The sporocarps were harvested, cleaned and packed in perforated, 100 gauge polypropylene cover (100 g of sporocarps in each cover), with ten holes (5 mm diameter) and stored in refrigerated condition (4 ^{0}C). Similarly, sporocarps were packed in polypropylene cover and stored at room temperature (26 ± 2 ^{0}C), as control experiment. Visual observations on the morphological characters and smell were recorded at 24 h interval. Per cent reduction in the weight of sporocarp was estimated, by periodically checking the weight of stored mushrooms at 1, 2, 3, 5, 10, 15, 20 and

30 days. Two separate experiments were carried out for *P. opuntiae* and *P. cystidiosus* (non-coremial), in CRD with 4 replications for each treatment (different days interval).

3.15. SENSORY EVALUATION

Equal quantity (100 g) of *P. cystidiosus* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous* were weighed and was cooked by sauting method. Steam cooked mushrooms were prepared with minimum salt and oil in order to record the time taken for cooking, development of off odour, colour change and to assess the real taste of mushrooms. Soup with mushroom pellets, bhaji and masala curry was also prepared, following the standard procedure as described in Appendix X. Organoleptic evaluation was performed with respect to six aspects *viz.,* colour, appearance, texture, flavour, taste and overall acceptability. Twelve post graduate students of the college were selected as the judging panel. Preference of the cooked sample was analysed using Hedonic rating scale as per the procedure described by Jellinick (1985). 9 point Hedonic rating scale (score card) is given in Appendix XI. The scores were tabulated and statistically evaluated using the Kruskal Wallis test.

3.16. POST HARVEST HANDLING AND ANALYSIS OF MICROFLORA

3.16.1. Sample preparation

Sporocarps of *P. cystidiosus* (non-coremial) and *P. opuntiae* were harvested in the morning and sample preparation was started immediately after harvest. Fresh mushroom samples, free from blemishes were washed thoroughly under running tap water, to remove the adhering soil particles, foreign materials, and to reduce the initial microbial load. The mushrooms were drained off water, sliced to pieces of 1cm thickness using stainless steel knife and divided into seven batches (500 g each) for various postharvest treatments.

3.16.2. Postharvest treatments

The postharvest treatments were (i) mechanical drying (ii) mechanical drying and powdering (iii) soaking in 1 per cent citric acid (CA) for 15 min followed by mechanical drying (iv) soaking in 1 per cent potassium metabisulphate (KMS) for 15 min followed by mechanical drying (v) soaking in 1 per cent CA for 15 min followed by mechanical drying and powdering (vi) soaking in 1 per cent KMS for 15 min followed by mechanical drying and powdering (vii) solar drying (control).

3.16.2.1. Mechanical drying

Cleaned and trimmed fresh mushrooms without pretreatments were spread on stainless steel trays and incubated in mechanical drier (TEMCO, Instruments and Equipments, Chennai) at 45-50 ^{0}C. The samples were dried until dry weight showed a constant value at consecutive intervals. The dried samples were cooled, immediately packed in polyethylene bag and stored at ambient condition. Similarly, mechanically dried sporocarps were powdered using mixer grinder separately packed in polyethylene bag and stored at ambient condition, for further studies.

3.16.2.2. Pretreatments followed by mechanical drying

The pretreatment of oyster mushrooms were carried out as suggested by Sugunal *et al.* (1995). The salt solutions *viz.*, CA and KMS were separately prepared at concentration of 1 per cent by dissolving required amounts of salt in distilled water. The mushroom samples prepared in 3.16.1. were soaked in the salt solutions separately at ambient temperature for 15 min, ensuring full coverage of the slices. The ratio of mushroom slices to the chemical solution was fixed at 1:5 (weight by volume). Mushroom samples treated with chemicals were placed on a blotting paper to remove the excess water from the surface and incubated in mechanical drier (TEMCO, Instruments and Equipments, Chennai) at 45-50 ^{0}C. The samples were dried until dry weight showed a constant value at consecutive intervals. The dried samples were cooled, immediately packed in polyethylene bag and stored at ambient condition for further studies.

3.16.2.3. Pretreatments followed by mechanical drying and powdering

Pretreated mushroom samples followed by mechanical drying from 3.16.2.2. were powdered using a mixer grinder.

3.16.2.4. Solar drying

Cleaned and trimmed fresh mushrooms without pretreatments were spread on black polythene sheet and dried under solar radiation. The samples were covered with a very thin gauze to protect the slices from dust and other foreign materials. The samples were stirred routinely to ensure uniform drying. The samples were dried until dry weight showed a constant value at consecutive intervals. The dried samples were cooled, immediately packed in polyethylene bag and stored at ambient condition for further studies.

3.16.3. Evaluation of postharvest treatments based on total plate count method

The best postharvest method for *P. cystidiosus* (non-coremial) and *P. opuntiae* isolates from 3.16.2. was selected based on microbiological examination of the samples using total plate count (TPC) or serial dilution and plating technique method (Waksman, 1922). Microbiological enumeration (*viz.*, fungus and bacteria) of mushrooms was done at fixed intervals, *i.e.* after every 2 months, up to microbial degradation and expressed as colony forming unit per gram ($cfug^{-1}$). Two separate experiments (for *P. cystidiosus* and *P. opuntiae*) were laid out in CRD with three replications and seven treatments (post harvest treatments).

One g of treated mushroom sample was transferred into a 250 ml conical flask containing 100 ml of sterile water. The sample was mixed thoroughly and shaken for 20 min in a mechanical shaker. It represented a dilution of 10^{-2}. 1 ml from this dilution was pipetted out to 99 ml of sterile water under sterile conditions. Again the flask was kept on a shaker for 15 min to attain a dilution of 10^{-4}. One ml from this suspension was pipetted out in to sterile petri dishes containing autoclaved and cooled Martins Rose Bengal Agar medium, with gentle, constant swirling as per the pour plate method. The petri dishes were

incubated at 25 ± 2 ^{0}C for 3-4 days. For the isolation of bacteria, 10^{-6} dilution was prepared from the 10^{-4} dilution. One ml from 10^{-6} dilution was plated on nutrient agar medium following the pour plate method and the plates were incubated at 25 ± 2 ^{0}C for 2-3 days. After the incubation period, fungal and bacterial colonies were counted and enumerated as following (Appendix XII).

TPC (cfug^{-1}) = mean colony count x dilution factor

The data were statistically analysed and interpreted.

3.17. STRAIN IMPROVEMENT *VIA* PROTOPLAST FUSION

Interspecific hybridization was carried out between the isolates of *P. cystidiosus* (non-coremial) and *P. opuntiae*, using polyethylene glycol (PEG) mediated fusion/somatic hybridisation.

3.17.1. Barrage reaction between *P. cystidiosus* (non-coremial) and *P. opuntiae*

Barrage reaction *via* formation of large contact zone was used as a presumptive evidence for the sexual compatibility between *P. cystidiosus* (non-coremial) and *P. opuntiae* isolates, as suggested by (Mallick and Sikdar, 2014). The selected parental mycelia were inoculated at 2 cm distance (dual plate technique), on petri dish, containing PDA (pH 6.2) and maintained at 24 ± 1 ^{0}C for 5 days, until two mycelia formed a large contact zone. The experiment was carried out in triplicate and macro-morphology of contact zone/barrage reaction *viz.*, thick barrages, line transects, fluffiness of growth and pigmentation were observed.

3.17.2. Double selection strategy for somatic hybrids

In somatic fusion experiments, hybrids are identified by double selection strategy (Lalithakumari, 2000). *P. cystidiosus* (PC2, PNC1) and *P. opuntiae* (PO1) were tested for their tolerance or sensitivity to the fungicide carbendazim at

concentrations of 0.1, 0.5 and 1 m*M*. Also the potential of parental isolates to utilize vanillin, a degradation product of lignin at concentrations of 0.01, 0.02 and 0.05 per cent were analysed. The experiment was carried out in triplicate and protoplast fusants were screened/segregated from the parental self fusants based on the selected, biochemical marker characteristics *viz.,* carbendazim tolerance and vanillin utilization.

3.17.3. Optimization of protocol for protoplast isolation

Factors controlling the protoplast isolation of the *Pleurotus* spp. *viz*., osmotic stabilizer, concentration of lytic enzyme, mycelial age and duration of incubation of mycelia with lytic enzyme were standardised for the optimization of protoplast production.

3.17.3.1. Effect of mycelium age, osmotic stabilizers, enzymes and incubation of mycelia on protoplasts isolation

Mycelia of *P. cystidiosus* (non-coremial) and *P. opuntiae* were harvested from the 100 ml PD broth at 3, 4 and 5 days of incubation. Keeping the different age of incubation constant, protoplasts were isolated with different osmotic stabilizers *viz*., 0.6 *M* sucrose and 0.6 M potassium chloride (KCl) (prepared in 0.01 *M* sodium phosphate buffer pH 6.5), using different concentration of lysing enzyme *viz*., 20, 25 and 30 mg/ml, at different duration of incubation of mycelia *viz*., 1, 2, 3, 3.5, 4.5 and 5 h. Protoplasts released were monitored by removing aliquots of the reaction mixture aseptically at an interval of 30 min examined under compound microscope (Carl Zeiss Primo star, Germany) and quantified using haemocytometer. Six experiments (for *P. cystidiosus* (non-coremial) and *P. opuntiae*), at 3, 4 and 5 days age of incubation were separately carried out in CRD with three replications for each treatment. Once the optimal conditions for maximum protoplast production were established, protoplast isolation of *P. cystidiosus* and *P. opuntiae* were performed using the standardised age of

mycelium, osmotic stabilizer, enzyme consortium at the optimised concentration (mg/ml) and duration of incubation of mycelia as per the standard procedure.

3.17.3.2. Protoplast isolation

Protoplasts were isolated and purified from the mycelial cultures of parents *viz.*, *P. cystidiosus* (non-coremial) and *P. opuntiae* as per the standard procedure described by Lalithakumari (2000). Mycelial bits (8 mm diameter) from actively growing cultures of the *Pleurotus spp.* (6 days old) were inoculated separately in conical flasks (250 ml) containing sterile PD broth (100 ml) and incubated at 28 ± 2 ^{0}C on a conical flask shaker (Amstrong Biotech Research, Bangalore) at 120 rpm. The mycelia (100 mg) was harvested, at the standardised age of incubation by filtration through Whatman filter paper no. 1, washed twice with sterile water and washed twice with the standardised osmotic stabiliser (prepared in 0.1 *M* sodium phosphate buffer, pH 6.5) (Appendix XIII). The washed mycelium of *Pleurotus* spp. were aseptically and separately transferred to sterile centrifuge tubes containing 2 ml of enzyme mixture (at the optimised concentration), dissolved in the osmotic stabilizer (prepared in 0.1 *M* phosphate buffer, pH 6.0) and incubated at 26 ± 2 ^{0}C in a rocking shaker with 50 rpm (Rockymax, Tarsons, Kolkatta). The protoplasts released at the optimised incubation time were observed under compound microscope (Carl Zeiss Primo star, Germany) and quantified using haemocytometer. A consortium of commercial enzymes *viz.*, β-glucanase, cellulase, protease and chitinase named as the Lysing enzyme from *Trichoderma harzianum* (L1412 SIGMA Glucanex-lyophilized powder) was used for the protoplast isolation. The enzyme consortium was prepared in the optimum concentration followed by filter-sterilization using sterilized filter disc assembly. The parameters of protoplast isolation was standardised by conducting CRD experiment, with 4 replications and 9 treatments. Treatments were the number of protoplasts released, at different hours of incubation), at varied enzyme concentration and osmotic stabilisers used.

3.17.4. Protoplast purification

Protoplasts were isolated from *P. cystidiosus* (non-coremial) and *P. opuntiae* using the optimised protocol standardised from 3.17.3. Protoplasts were filtered from the hyphal debris, through a column of cotton wool packed up to the 0.5 ml mark of a 5 ml syringe. Protoplasts were then collected and sedimented from the filtrate by centrifugation at 2000 rpm for 15 min. Sedimented protoplasts were washed twice in the best osmotic stabiliser and suspended in 5 ml of the same osmotic stabilizer. The purified protoplasts were further used for the subsequent experiments.

3.17.5. Regeneration of protoplasts

Regeneration of protoplasts of *P. cystidiosus* (non-coremial) and *P. opuntiae* were checked in solid medium *viz.*, malt extract, yeast extract, glucose medium (MYG) (Appendix XIII) (Mukherjee and Sengupta, 1987). The purified protoplasts were diluted with the standardised osmotic stabiliser to about 10^4 cells/ml and 0.1 ml of the diluted suspension was plated on petri dishes containing 25 ml of MYG with standardised osmotic stabilizer (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) as per the pour plate method. The plates were incubated at 28 ± 2 ^{0}C for 3-4 days. Similarly, an equal volume of protoplast suspension was mixed with equal volume of distilled water and plated on the osmotically stabilised MYG medium served as control. In the control plate, all the protoplasts bursted and no mycelial colonies appeared on the plates, unless some mycelial fragments got included in the sample. Number of colonies which appeared on the regeneration medium was counted and regeneration frequency was assessed. Regeneration frequency referred to the fraction of protoplasts that regenerated a new cell wall and reverted to normal hyphal growth.

$$\text{Regeneration frequency} = \frac{\text{Number of colonies appeared on regeneration medium}}{\text{Number of protoplasts plated}}$$

3.17.6. PEG mediated fusion

The PEG-mediated fusion was carried out according to Anne and Peberdy (1975) with some modifications. The purified protoplasts of *P. cystidiosus* (non-coremial) and *P. opuntiae* were separately diluted to $1x10^4$ protoplasts ml^{-1}, in the best osmotic stabilizer (prepared in 0.01 *M* sodium phosphate buffer pH 6.5). Freshly prepared, diluted protoplast suspension of the parents (1 ml each) were mixed in sterile centrifuge tube and 1 ml of fusion mixture was added to the purified parental protoplasts. The mixture was incubated at room temperature for 20 min, by shaking the tube every 5 min manually for uniform mixing. Fusion mixture comprised of polyethyleneglycol (PEG) (molecular weight 4000, 30 per cent), calcium chloride (0.05 *M*) and glycine (0.05 *M*). After uniform mixing, the mixture was centrifuged at 2000 rpm for 10 min and supernatant was decanted. Pelleted protoplasts were washed twice with the osmotic stabilizer and resuspended in 5 ml of osmotic stabilizer. The fusion processes were examined by observing the aliquots of fusion suspensions (10 µl), under a high resolution compound microscope (Carl Zeiss Primo star, Germany) at 40 and 100 magnifications.

3.17.7. Regeneration of fused protoplasts

Malt-yeast extract glucose medium (MYG) osmotically stabilised with the best osmotic stabiliser (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) was used as the regeneration medium for the protoplast fusants (Mukherjee and Sengupta, 1987). 100 µl of the PEG treated, presumptively fused protoplasts was plated onto petri dishes containing osmotically stabilised regeneration media (MYG) amended with the dual biochemical markers *viz.*, carbendazim and vanillin at the standardised concentrations from 3.17.2. Petri dishes were incubated at 25 ^{0}C for the development of presumptive (putative) somatic hybrid colonies (macrocolonies). After 1-2 days, somatic hybrid colonies were formed on the surface of the amended MYG medium (containing dual biochemical markers)

and were individually sub-cultured on fresh PDA slants for subsequent studies. The purity of the protoplast suspension was checked by maintaining a control plate wherein the suspension of protoplasts initially lysed with distilled water was plated in similar way. The absence of mycelial colonies on the control plate was taken as a measure of the purity of protoplast. Fusion frequency was determined as the ratio of number of colonies which appeared on regeneration medium to the number of protoplasts plated. Fusion frequency of fusants was compared with the regeneration frequency of *P. cystidiosus* (non-coremial) and *P. opuntiae* from 3.17.5. based on CRD experiment with five replications for each treatment (isolates).

3.17.8. Confirmation and evaluation of protoplast isolates and fusants

3.17.8.1. Comparison of radial colony growth and hyphal width of protoplast isolates, fusants and parents

Protoplast isolates (2 strains) and fusants (8 strains) were evaluated for the evidence of variability by comparing their radial colony growth and nature of mycelial growth with the parental strains (*P. cystidiosus* non-coremial and *P. opuntiae*) on the best medium screened from 3.5.1. Width of the hyphae was also compared, using compound microscope (Carl Zeiss Primo star, Germany) under 10 X and 40 X magnifications. The experiment was carried out in CRD with three replications and twelve treatments. Treatments were 2 isolates, 8 fusants and 2 parental isolates. The data was statistically analysed and interpreted.

3.17.8.2. Comparative spawn production studies with protoplast isolates, fusants and parents

Spawns of protoplast isolates (2 strains) and fusants (8 strains) were compared with the parental strains using the best spawn medium amended with the best amendment screened in 3.8.4 as per the standard procedure. Observations on the time taken for spawn run, nature of mycelial growth and shelf life were

recorded in case of spawn production for all the evaluated oyster mushroom isolates. The experiment was carried out in CRD with three replications and twelve treatments. The treatments were spawns of 2 isolates, 8 fusants and 2 parental isolates. The data was statistically analysed and interpreted.

3.17.8.3. Cultivation trials with protoplast isolates, fusants and parents

Cultivation trials of protoplast isolates (2 strains), fusants (8 strains) and parental isolates were conducted during the September-February season of 2017-2018 using the bed substrates *viz.,* paddy straw and rubber wood sawdust as per the standard polybag method under indoor climatic conditions in the growing rooms. The developmental morphology, total yield, total crop period, average weight of sporocarp, number of sporocarps and B.E were recorded during the process of mushroom production. The experiment was carried out in CRD with three replications and twelve treatments. Twelve treatments were 2 isolates, 8 fusants and 2 parental isolates. The data was statistically analysed and interpreted. Also cultivation trials with the second generation of promising fusants *viz*., F4, F6 and F8 were carried out in CRD experiment with 4 replications. The parent isolates served as control.

Results

4. RESULTS

Pleurotus commonly known as 'oyster mushroom' belong to the Phylum:- Basidiomycotina; Class:-Basidiomycetes; Subclass:- Holobasidiomycetidae; Order:- Family:-Polyporaceae; Genus:- *Pleurotus*. There are species of *Pleurotus* found suitable for cultivation *viz.*, *sajor caju, sapidus, ostreatus, eous, membranaceous, florida, citrinopileatus, flabellatus, pulmonarius, geesteranus, ulmarius, tuberregium, cystidiosus* and *eryngii.* In Kerala *P. florida*, *P. sajor caju* and *P. eous* are the widely cultivated and appreciated species. The occurrence of two new species of *Pleurotus viz., cystidiosus* and *opuntiae* in Kerala have been reported by Geetha (2011; 2015). Preliminary trials conducted with these two species at College of Agriculture, Vellayani indicated the scope for their large scale production (Geetha, 2015). However, no systematic studies have been done on the large scale production of these species and hence the present study will help for the introduction of two new species in Kerala. Apart from the usual methods of breeding (introduction and selection) somatic hybridization through protoplast fusion is a best way to combine genetic characters across the species barrier. In this regard an investigation was also undertaken on the strain improvement through protoplast fusion of oyster mushrooms *viz.*, *P. cystidiosus* and *P. opuntiae* and the results of the study are presented below.

4.1. SURVEY

Random surveys were conducted purposively in locations having comparatively dense vegetations and observations during these surveys were recorded based on the data sheet given by Nair (1990) (Appendix I). Mushrooms were collected from two different locations of Thiruvananthapuram *viz.*, Vellayani and Chirayinkeezhu to identify the indigenous *P. cystidiosus* and *P. opuntiae* growing under natural conditions during pre and post monsoon showers of 2015-2017. The details of the survey are given in Table 1 and 2.

In Vellayani, Ashoka (*Saracca indica)* and Eucalyptus (*Eucalyptus globus*) logs were found to be hosts for the coremial isolate of *P. cystidisous* (Plate 1). The non-coremial isolate of *P. cystidiosus* was collected only from Chirayinkeezhu and was found growing on fallen wood logs of mango (*Mangifera indica*). A foreign non-coremial isolate of *P. cystidisous* was developed from dowel spawn procured from Germany (Plate 2). In Vellayani, mango (*Mangifera indica* L) logs, coconut (*Cocos nucifera* L.) and arecanut (*Areca catechu L.*) logs were found to be the hosts for *P. opuntiae* (Durieu and Lev.) (Plate 3). All the collected mushrooms were gregarious and lignicolous in nature, except isolate number three of *P. opuntiae* from Vellayani, which was solitary in nature.

4.2. MACROSCOPIC STUDIES

Macroscopic characters *viz.*, colour, shape and texture of pileus, length of stipe, colour of gills, gill number per cm and presence of volva/annulus of the isolates of *Pleurotus* spp. were studied in detail. The descriptions are given in Table 1 & 2 and Plate 4.

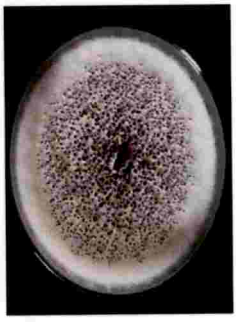
4.3. ISOLATION AND PURE CULTURING

The collections of *Pleurotus* spp. obtained during the survey were isolated to obtain pure culture of the fungus at the Department of Plant Pathology, College of Agriculture, Vellayani. Partially matured, pests and disease free (*Pleurotus* spp.) sporocarps obtained during the survey were selected for isolation which was done by tissue culturing as per the standard method (Suharban, 1987).

Two days after inoculation, white hair like strands of mycelial growth started growing from the tissues of *P. cystidiosus* (non-coremial) and *P. opuntiae* inoculated on PDA medium, whereas, *P. cystidiosus* (coremial) took 7 days after inoculation. *P. cystidiosus* (non-coremial) showed white, thick and fluffy growth; and took 11 days for the completion of growth. *P. cystidiosus* (coremial) had

Table 1. Morphological characters of isolates of *P. cystidiosus* (coremial and non-coremial)

Isolate	Locations	Habit	Habitat	Host plant	Sporocarp		Pileus size (lengthxbreadth-cm)	Texture of pileus	Gills type, no./cm	Stipe type, length (cm)	Volva and Annulus	Date of collection, min/max temp.(^{0}C), min/max RH (%)
					Colour	Shape						
Isolate 1	Vellayani (coremial)	Gregarious (cluster of 8)	Lignicolous	Ashoka *(Saracca indica)*	Light brown	Oyster shaped, fringed margin	19.70 x 16.51	Smooth	Free and fleshy, 9 gills/cm	Lateral and fleshy, 3.12 cm	Absent	28.05.2016 24.4 /32.8 ^{0}C 77/92 % RH
Isolate 2	Vellayani (coremial)	Gregarious (cluster of 4)	Lignicolous	Eucalyptus (*Eucalyptus globulus*)-logs	Creamish white	Oyster shaped, fringed margin	18.33 x 15.40	Smooth	Free and fleshy, 11 gills/cm	Lateral and fleshy, 2.80 cm	Absent	27.06.2016, 24.6/31.3 ^{0}C, 87/97.5 % RH
Isolate 1	Chirayin-keezhu (non-coremial)	Gregarious (cluster of 3)	Lignicolous	Mango (*Mangifera indica*)-logs	White	Oyster shaped, smooth margin	15.00 x 11.52	Smooth	Free and fleshy, 8 gills/cm	Lateral and fleshy, 4.00 cm	Absent	12.12.2016 23.7/31.6 ^{0}C 91/93 % RH



Isolate 1:PC1 **Isolate 2: PC2**

Plate 1. *P. cystidiosus* (coremial) mushrooms from Vellayani

Mycelial culture

Sporocarps

a. From *Areca catechu* L. logs

Dowel spawn

Mycelial culture

Sporocarps

b. From commercial dowel spawn

Plate 2. Isolation of *P. cystidiosus* (non-coremial)

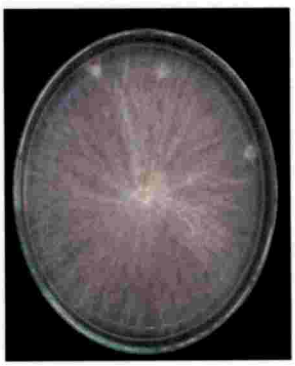
Table 2. Morphological characters of isolates of *P. opuntiae*

Isolate	Location	Habit	Habitat	Host plant	Sporocarp		Pileus size (lengthxbreadth-cm)	Texture of pileus	Gills type, no./cm	Stipe type, length (cm)	Volva and annulus	Date of collection, min/max temp.(^{0}C), min/max RH (%)
					Colour	Shape						
Isolate 1	Vellayani	Gregarious (cluster of 7)	Lignicolous	Mango (*Mangifera indica* L.)-logs	White	Oyster shaped, fringed margin	5.10 x 4.02	Smooth	Free and fleshy, 6 gills/cm	lateral and fleshy, 0.40 cm	Absent	28.05.2016, 24.4 /32.8 ^{0}C, 77/92 % RH
Isolate 2	Vellayani	Gregarious (cluster of 3)	Lignicolous	Coconut (*Cocos nucifera* L.),	Creamish white	Oyster shaped, fringed margin	9.80 x 5.35	Smooth	Free and fleshy, 8gills/cm	lateral and fleshy, 0.45 cm	Absent	27.06.2016, 24.6/31.3 ^{0}C, 87/97.5 % RH
Isolate 3	Vellayani	Solitary	Lignicolous	Arecanut (*Areca catechu* L.)-logs	White	Oyster shaped, fringed margin	8.26 x 4.98	Smooth	Free and fleshy, 7 gills/cm	rudimentary stipe	Absent	5-5-2016, 24.3/35.6 ^{0}C, 78/95% RH

a. Isolate 1. PO1

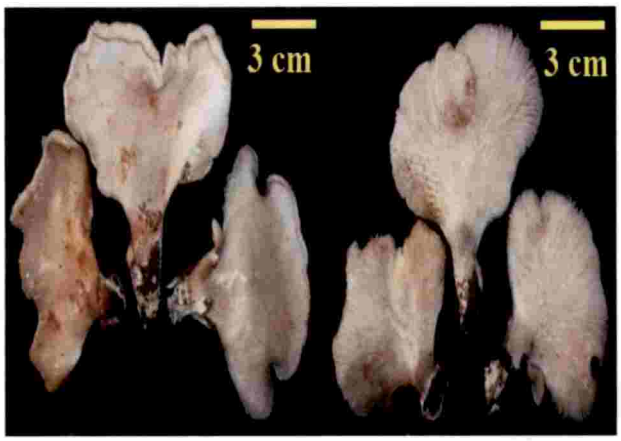
b. Isolate 2. PO2

c. Isolate 3. PO3

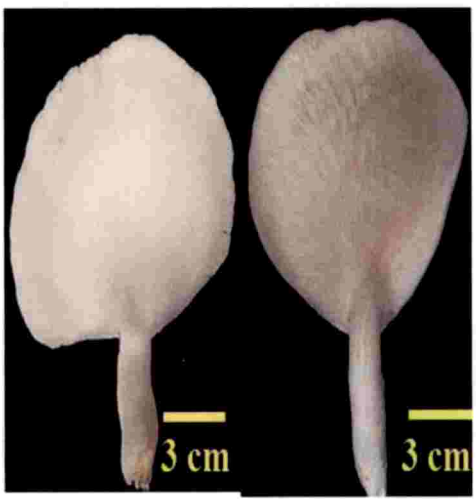


Mycelial culture

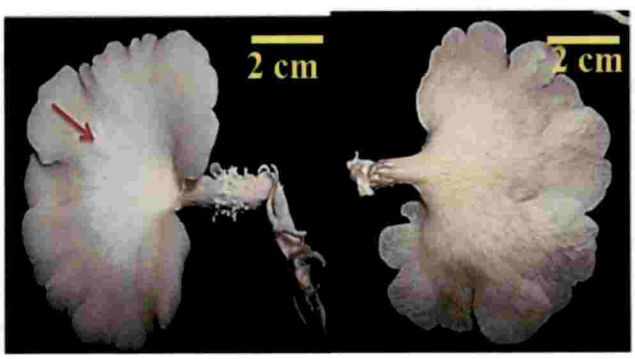
Plate 3. *P. opuntiae* mushrooms from Vellayani



a. *P. cystidiosus* (coremial)



b. *P. cystidiosus* (non-coremial)



Dorsal side Ventral side

c. P. opuntiae

Plate 4. Morphological characters of sporocarps

white, thin, cottony growth with coremia and took 50 days for complete coverage of test tube. Mycelium of *P. cystidiosus* (coremial) showed the development of small protuberances on the entire tissue followed by the appearance of little white stalk and tiny watery droplets having blackish colour. As the growth continued the mycelium of *P. opuntiae* turned white, thin, cottony and branched. The pure cultures of seven collections *viz.*, two coremial and two non-coremial isolates of *P. cystidiosus*; and three isolates of *P. opuntiae* were maintained, sub cultured and designated as PC1, PC2, PNC1, PNC2, PO1, PO2 and PO3 respectively.

4.3.1. Selection of fast growing isolates of *Pleurotus* spp.

Observations on days taken for the growth of cultures of *Pleurotus* spp. were recorded. The values were statistically analysed to screen the fast growing isolates from the collections obtained during the survey.

Among the coremial isolates of *P. cystidiosus*, PC2 took minimum number of days (29.50 days) for complete coverage on petri dish followed by PC1 (39.30 days) (Table 3). The isolate 2 recorded thick and fluffy growth with smooth margin whereas, isolate 1 showed thin and sparse growth with radiating margin. Among the non-coremial isolates of *P. cystidiosus*, PNC1 recorded the minimum time for complete coverage on petridish (5.94 days) followed by PNC2 (6.12 days) (Table 4). The former isolate recorded thick and fluffy growth with concentric zonations and radiating margin whereas, the later isolate showed thin and diffused growth. Among the isolates of *P. opuntiae,* PO1 recorded the minimum time for complete coverage on petridish (6.80 days) followed by PO2 (8.00 days) and PO3 (10.60 days) (Table 5). The nature of mycelial growth of PO1 and PO3 was thin with radiating margin whereas, PO2 had thin mycelium with concentric zonations and radiating margin. The screened isolates of *P. opuntiae* (PO1) and *P. cystidiosus* (coremial-PC2) were identified as *P. opuntiae* and *P. cystidiosus.* Also accession numbers *viz.*, DMRO-925 for PO1 and DMRO-926 for PC2, were retrieved from Directorate of Mushroom Research (DMR), ICAR, Solan, Himachal Pradesh.

4.4. MICROSCOPIC STUDIES

Microscopic characters of hyphae, spores and cystidia of the fast growing isolates of *Pleurotus* spp., *viz*., *P. cystidiosus* (coremial), P. cystidiosus (non-coremial) and *P. opuntiae* were studied (Table 6). Hyphae of *Pleurotus* spp. were septate, branched, hyaline, aerial as well as submerged with distinct clamp connection (Plate 5). Hyphae of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* recorded sizes of 6.86, 2.49 and 1.65 µm respectively. Spore print of the three isolates were of pure white in colour (Plate 6). Cystidia are sterile structures present between the basidia. Cheilocystidia are found on the edge of the lamella (analogous hymenophoral structure) whereas, pleurocystidia are seen on the face of the lamella. Pleurocystidia of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* recorded sizes of 41.70 µm x 3.91 µm, 17.99 µm x 3.17 and 16.92 µm x 2.85 µm, respectively, whereas cheilocystidia recorded sizes of 41.48 µm x 2.11 µm, 43.63 µm x 6.13 µm and 32.84 µm x 2.97 µm respectively (Plate 7). Basidiospores of *Pleurotus* spp. were hyaline, oval, sub cylindrical to cylindrical and they stained dark blue in lactophenol cotton blue indicating positive cyanophilic reaction (cyanophilic spores). When stained with 1 per cent congo red, lipid distribution was distinctly seen as black inclusions inside the basidiospores (Plate 8). Basidiospores of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* recorded sizes of 4.36 µm x 1.83 µm, 20.24 µm x 5.85 µm and 8.75 µm x 3.64 µm respectively.

4.5. MOLECULAR CHARACTERIZATION OF *PLEUROTUS* SPP.

4.5.1. Polymerase chain reaction (PCR)

Molecular diagnosis using PCR and ITS specific primers followed by gel electrophoresis was performed for the detection of the three screened *Pleurotus* spp. (from 4.3.1.) along with the two ruling mushrooms of Kerala *viz*., *P. florida* and *P. eous*. PCR amplification of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous* gave amplicons of sizes 656, 577, 657, 631 and 637 bp respectively (Plate 9).

Table 3. Selection of fast growing isolate of *P. cystidiosus* (coremial)

Sl. No.	Isolates	Nature of mycelial growth	*Time for complete coverage on petri dish (days)
1.	PC1	Thin and sparse with radiating margin	39.30
2.	PC2	Thick and fluffy with smooth margin	29.50
t (0.05)		2.101**	

*Average of ten replications

** Treatments are significantly different at 5 per cent level

Table 4. Selection of fast growing isolate of *P. cystidiosus* (non-coremial)

Sl. No.	Isolates	Nature of mycelial growth	*Time for complete coverage on petri dish (days)
1.	PNC1	Thick and fluffy with concentric zonations and radiating margin	5.94
2.	PNC2	Thin and diffused	6.12
t (0.05)		2.101**	

*Average of ten replications

** Treatments are significantly different at 5 per cent level

Table 5. Selection of fast growing isolate of *P. opuntiae*

Sl. No.	Isolates	Nature of mycelial growth	*Time for complete coverage on petri dish (days)
1.	PO1	White, thin, sparse with radiating margin	6.8
2.	PO2	White, thin with concentric zonations and radiating margin	8.0
3.	PO3	White, thin, sparse with radiating margin	10.6
SE m (±)		1.121	
CD (0.05)		0.974	

* Average of five replications

Means followed by similar superscripts are not significantly different at 5 per cent level

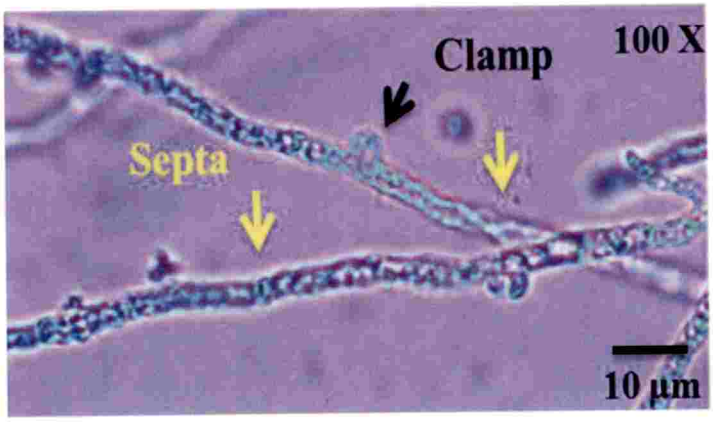
Table 6. Microscopic studies on *Pleurotus* spp.

Sl. No.	Characters	*P. cystidiosus* (coremial)	*P. cystidiosus* (non-coremial)	*P. opuntiae*
1	Hyphae	6.86 µm	2.49 µm	1.65 µm
2	Spore shape	Oval	Oval	Oval
3	Spore size	4.36 x 1.83 µm	20.24 x 5.85 µm	8.75 x 3.64 µm
4	Shape and size of pleurocystidia	Club shaped, 41.70 x 3.91 µm	Club shaped, 17.99 x 3.17 µm	Club shaped, 16.92 x 2.85 µm
5	Shape and size of cheilocystidia	Club shaped, 41.48 x 2.11 µm	Tubular, 43.63 x 6.13 µm	Club shaped, 32.84 x 2.97µm
6	Spore print	White	White	White

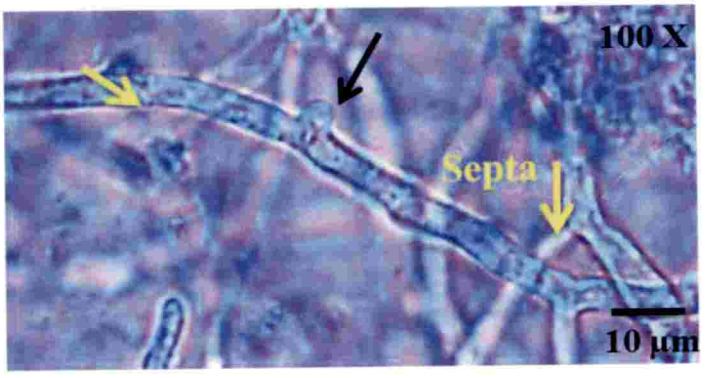
Table 7. Accession numbers from NCBI

Sl. No.	Isolates	*Pleurotus* species	Accession numbers
1	PO1	*Pleurotus opuntiae*	KY214255
2	PC2	*Pleurotus cystidiosus* subsp. *abalonus*	KY214254
3	PNC1	*Pleurotus cystidiosus*	KY887023
4	PE	*Pleurotus eous*	KY214257
5	PF	*Pleurotus florida*	KY214256

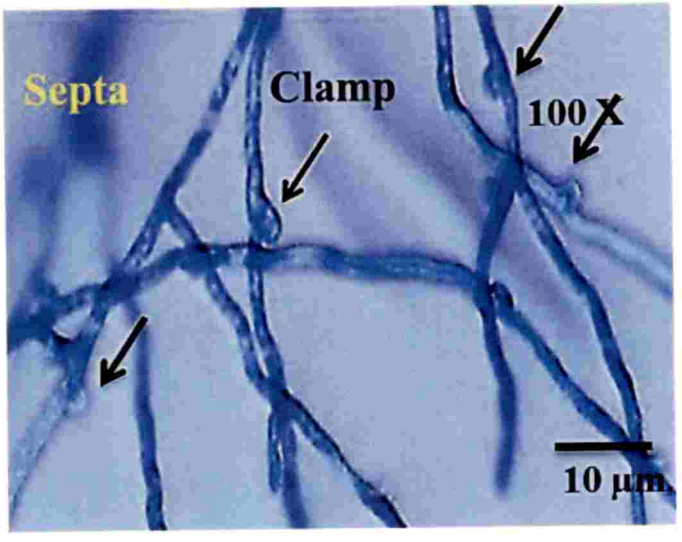
*NCBI: National centre for biotechnological information



a. *P. cystidiosus* (coremial)



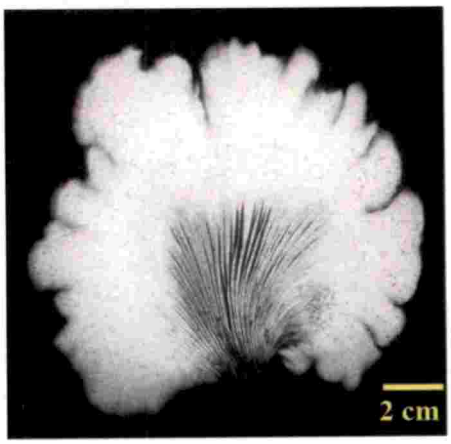
b. *P. cystidiosus* (non-coremial)



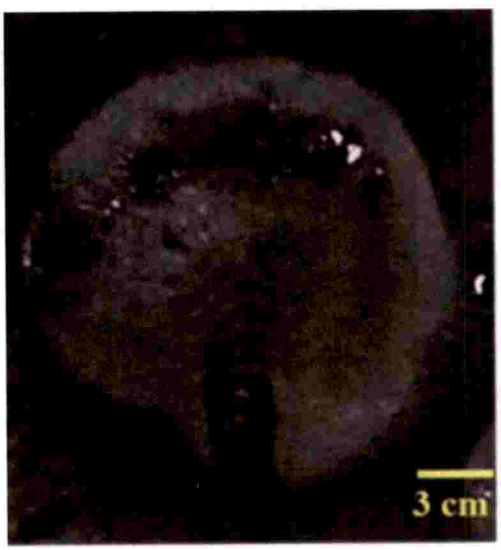
c. *P. opuntiae*

Plate 5. Hyphae of *Pleurotus* spp.

a. *P. cystidiosus* (coremial)

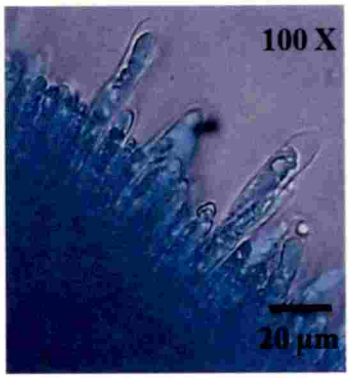


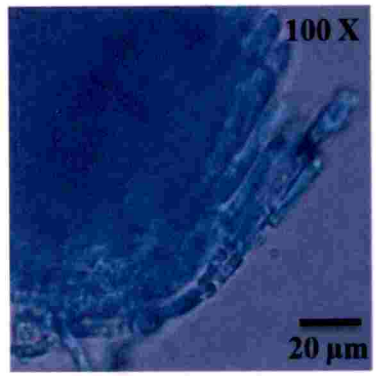
b. *P. opuntiae*



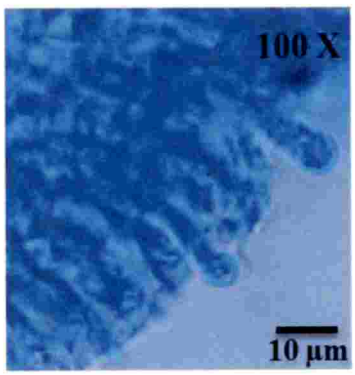
c. *P. cystidiosus* (non-coremial)

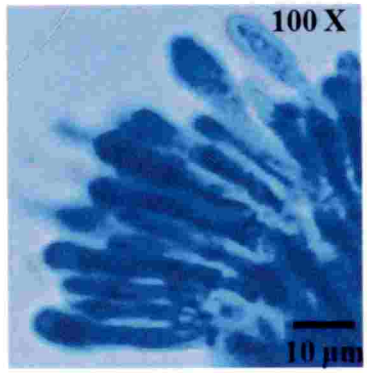
Plate 6. Spore print of *Pleurotus* spp.



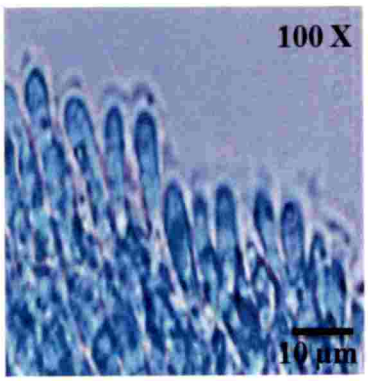


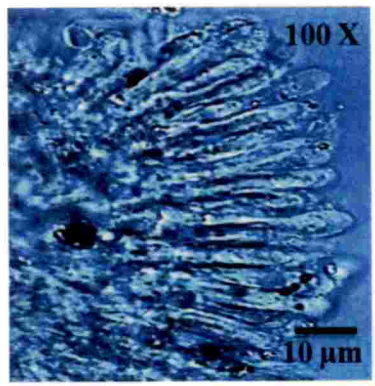
a.P. cystidiosus **(coremial)**





b.P. cystidiosus **(non-coremial)**





Pleurocystidia **Cheilocystidia**

c.P. opuntiae

Plate 7. Cystidial characters of *Pleurotus* spp.

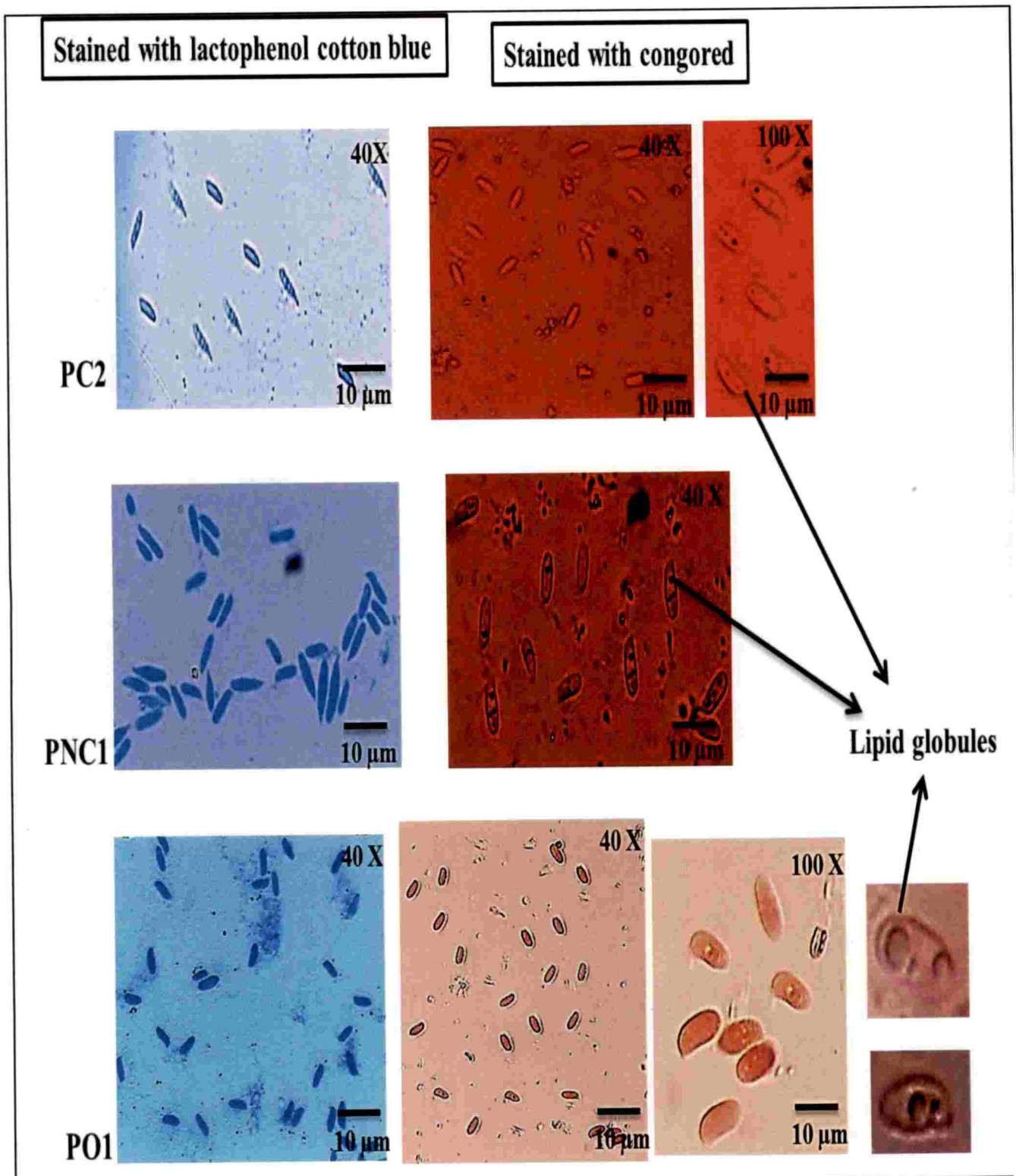


Plate 8. Basidiospores of *Pleurotus* spp.

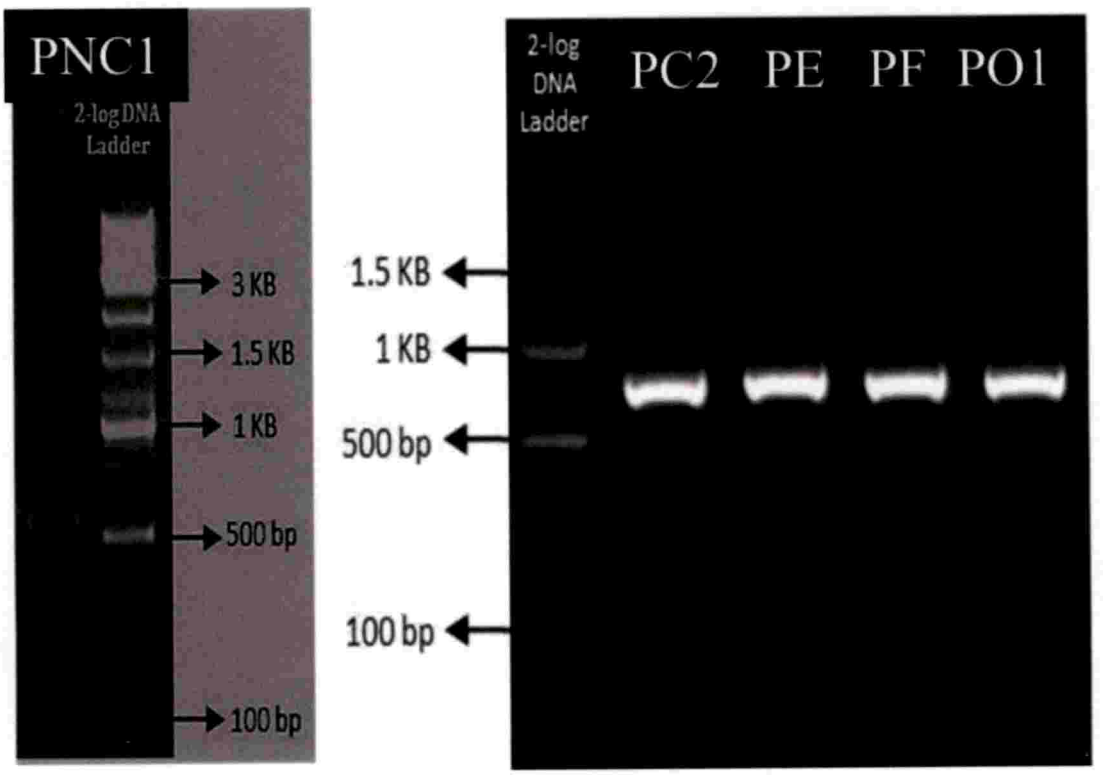


Plate 9. PCR amplification of *Pleurotus* spp.

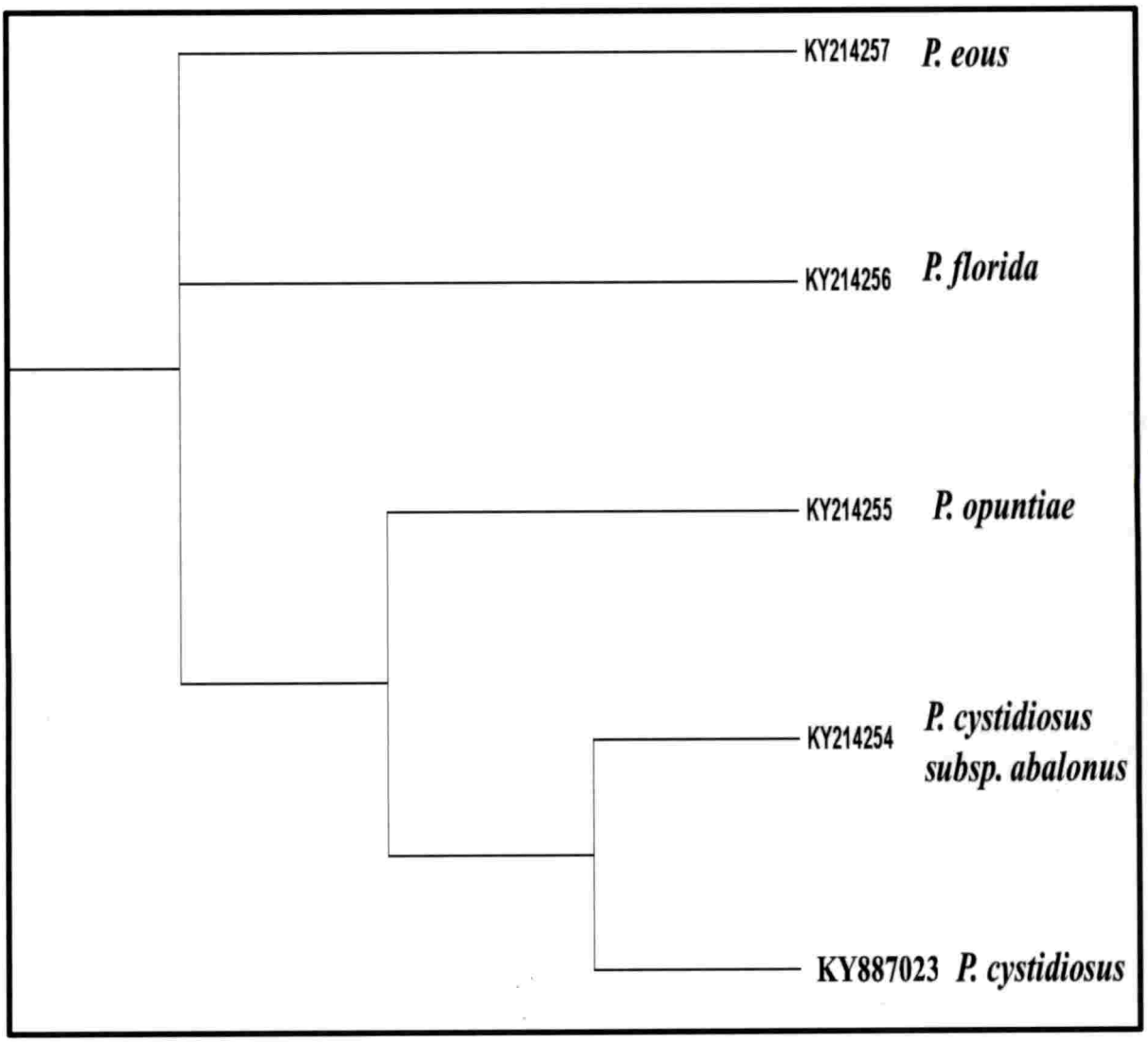


Fig 1. Phylogenetic tree showing the relationship of *Pleurotus* spp.

4.5.2. Characterisation

The PCR products obtained were sequenced and sequences were identified using BLAST software. Comparative nucleotide sequence alignment of the respective *Pleurotus* spp. with the available data bases from NCBI were done (Table 7). *P. cystidiosus* (coremial) shared 100 per cent homology with *P. cystidiosus* subsp. *abalonus* strain from USA and Korea. *P. cystidiosus* (non-coremial) showed 100 per cent homology with *P. cystidiosus* from China. *P. opuntiae* shared 100 per cent homology with isolates of *P. opuntiae* from China, USA and Gujarath; and 98 per cent homology with isolates of *P. djamor* from Brazil and China. Thus the three isolates were identified as *P. cystidiosus* subsp. *abalonus*, *P. cystidiosus* and *P. opuntiae* respectively. Comparative nucleotide sequence alignment was also carried out among the five *Pleurotus* spp. and dendrogram was drawn. *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* came under a larger cluster and was distantly related to *P. florida* and *P. eous*. *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous* were deposited in the Genbank NCBI data base and accession numbers *viz.*, KY214254, KY887023, KY214255, KY214256 and KY214257 were retrieved.

4.6. PHYSIOLOGICAL STUDIES OF PLEUROTUS SPP.

The fast growing screened isolates of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were used in the subsequent studies of the investigation.

4.6.1. Growth of *Pleurotus* spp. in different media

Cultural characteristics of fast growing isolates *viz.*, *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were studied on four different media. The results are presented in Table 8.

Table 8. Mycelial growth of *Pleurotus* spp. on different media

Sl. No.	Media	Nature of mycelial growth			*Days for complete coverage on petri dish		
		P. cystidiosus coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	P. *opuntiae*
1.	PDA	White, thin, with radiating margin	White, thin with radiating margin	White, thin with radiating margin	30.75^{c}	9.25^{b}	6.40^{b}
2.	OMA	White, thin, with branched margin	White, thin with concentric zonations and radiating margin	White, thin, sparse with radiating margin	40.75^{a}	7.25^{c}	7.20^{a}
3.	CEA	Light brown, thin with radiating margin	White, thin with concentric zonations and radiating margin	White, thin, sparse, with concentric zonations and radiating margin	36.00^{b}	6.00^{d}	7.40^{a}
4.	MEA	White, thick, fluffy with radiating margin	White, thin, restricted	White and thick with concentric zonations and radiating margin	29.25^{d}	15.00^{a}	6.20^{b}
5.	PDPA	White, thick, fluffy with radiating margin	White, thick cottony with concentric zonations and radiating margin	Cottony white, thick, fluffy with radiating margin	29.50^{d}	5.75^{d}	6.00^{b}
SE m (±)					2.237	1.704	0.278
CD (0.05)					1.045	0.976	0.590

*Average of five replications, Means followed by similar superscripts are not significantly different at 5% level, PDA: Potato dextrose agar medium, OMA: Oat meal extract agar, CEA: Carrot extract agar medium, MEA: Malt extract agar medium, PDPA: Potato dextrose peptone agar medium

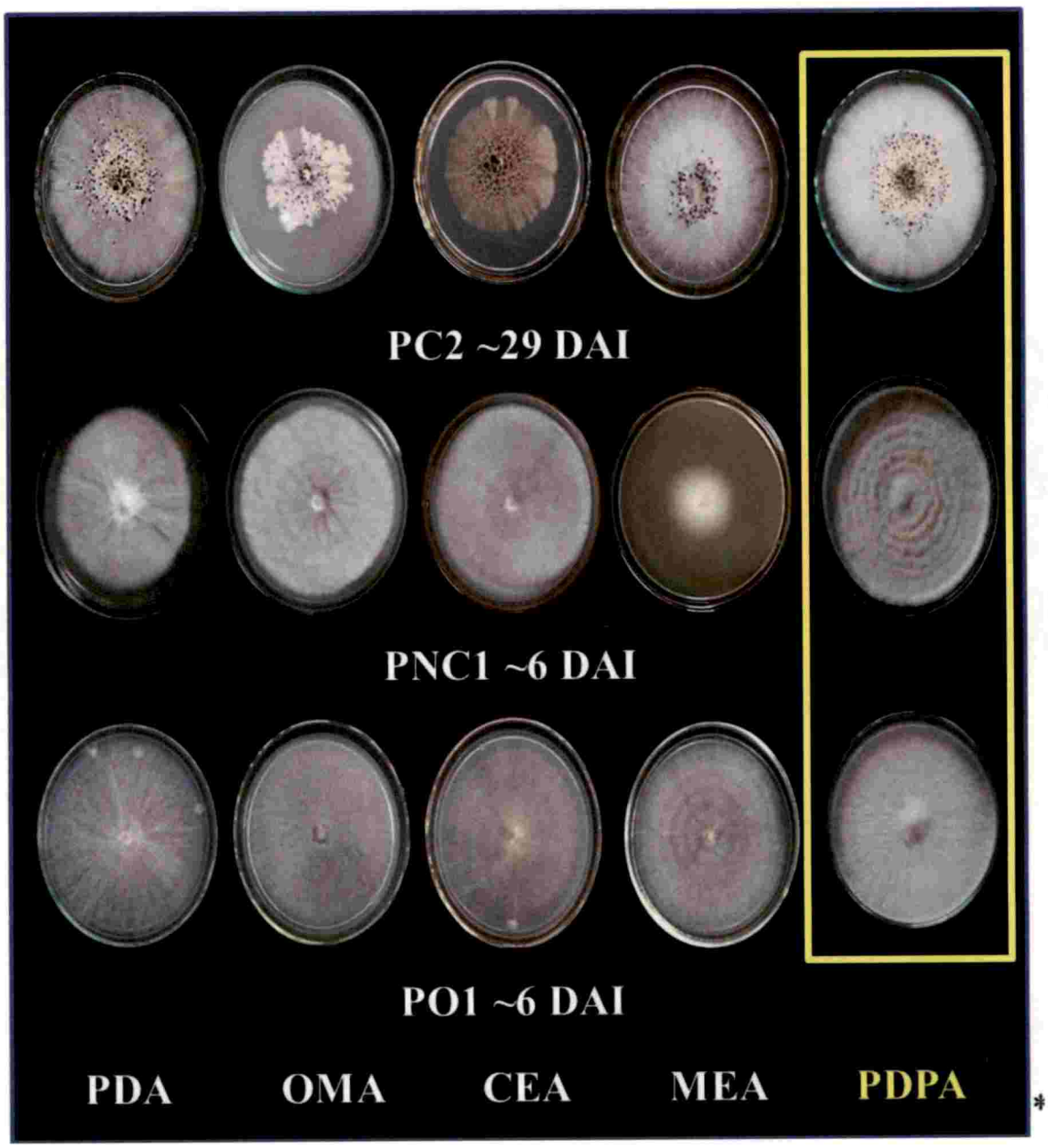


Plate 10. Influence of media on mycelial growth of *Pleurotus* spp.

P. cystidiosus (coremial) took the minimum time for completing the mycelial growth in MEA (29.25 days) and PDPA (29.50 days). The next best results were obtained on PDA (30.75 days) and CEA (36.00 days). White, thick and fluffy growth with radiating margin was seen in PDPA and MEA whereas, thin growth was observed with the other three media. *P. cystidiosus* (non-coremial) took the minimum time for completing the mycelial growth on PDPA (5.75 days) and CEA (6.00 days), followed by OEA (7.25 days) and PDA (9.25 days). White, thick, cottony growth with concentric zonations and radiating margin was observed in PDPA whereas, white, thin, growth was observed with the other four media. The results revealed that among the different media tested for the growth of *P. opuntiae*, PDPA (6.00 days), MEA (6.20 days) and PDA (6.40 days) took the minimum time for complete coverage of petridish followed by OEA (7.2 days) and CEA (7.4 days). However, cottony white, thicker and fluffy growth with radiating margin and initiation of primordia was recorded in PDPA medium. Hence PDPA was screened as the best medium for *P. opuntiae* based on the minimum number of days taken for complete coverage of petriplate and thickness of mycelial growth. Thus PDPA was identified as the best medium for the three isolates (Plate 10).

4.6.2. Growth of *Pleurotus* spp. at different temperature

The physiological characteristics of isolates *viz., P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were studied at three different temperature levels and the results of the study are presented in Table 9. *P. cystidiosus* (coremial) took the minimum time for completion of growth at 30 ^{0}C (29.days) followed by 25 ^{0}C (34.75 days) and 20 ^{0}C (36.50 days). However, at 35 ^{0}C no mycelial growth was observed. White, thick growth with radiating margin was observed at 30 ^{0}C. Thick, fluffy growth with branched margin was seen at 25 ^{0}C and thin, sparse growth was observed at 20 ^{0}C. *P. cystidiosus* (non-coremial) took the minimum time for completion of growth at 30 ^{0}C (5.75 days) and 25 ^{0}C (6.50 days) followed by 20 ^{0}C (9.50 days). However, at 35 ^{0}C no mycelial growth

Table 9. Influence of temperature on mycelial growth of *Pleurotus* spp.

Sl. No.	Temperature (^{0}C)	Nature of mycelial growth			*Days for complete coverage on petri dish		
		P. cystidiosus coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	*P. opuntiae*
1	20	Thin, sparse	White, thick, cottony with concentric zonations	Thin, sparse, slow	36.50[a]	9.50[a]	10.50[a]
2	25	Thick, fluffy, with branched margin	White, thick, cottony, concentric	Thick, white, fluffy with radiating margin	34.75[b]	6.50[b]	7.25[b]
3	30	White, thick with radiating margin	White, thick, cottony with concentric zonations	White, thin with radiating margin	29.25[c]	5.75[b]	6.50[b]
4	35	No growth	No growth	No growth	-	-	-
SE m (±)					2.184	1.145	1.227
CD (0.05)					0.849	0.881	0.881

* Average of five replications
Means followed by similar superscripts are not significantly different at 5 % level
Treatments without observation not taken for statistical analysis

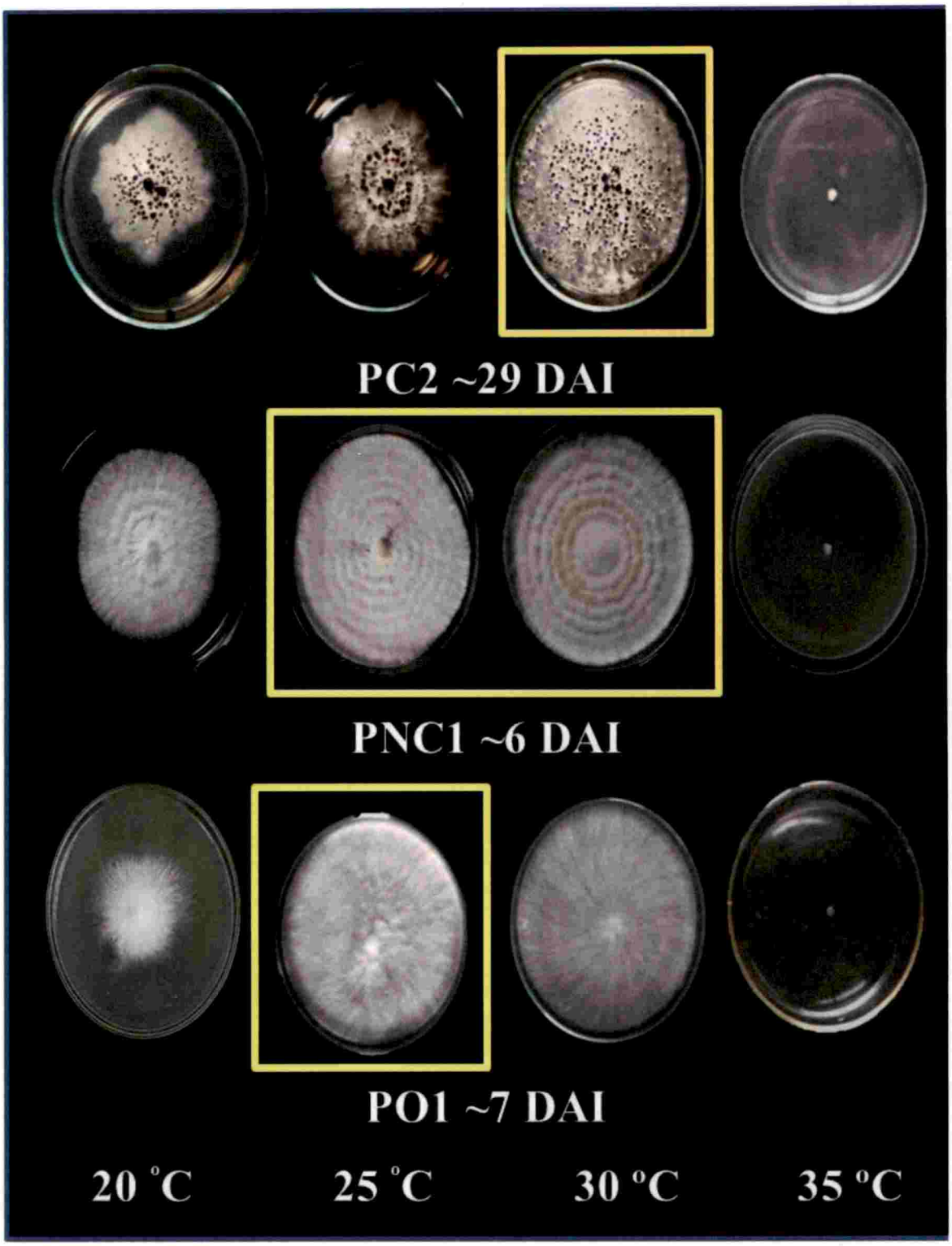


Plate 11. Influence of temperature on mycelial growth of *Pleurotus* spp.

was observed. White, thick cottony growth with concentric zonations was observed at 20, 25 and 30 ^{0}C. Among the different temperature levels tested for *P. opuntiae*, temperatures of 30 and 25 ^{0}C were found to be the best and took minimum time of 6.50 and 7.25 days to complete mycelial growth followed by 20 ^{0}C (10.50 days), along with the initiation of primordia. Absence of growth was observed at 35 ^{0}C. However, thick, white, fluffy growth with radiating margin was observed at 25 ^{0}C whereas, white, thin, growth with radiating margin was recorded at 30 ^{0}C. Hence 25 ^{0}C was identified as the best temperature for *P. opuntiae*. Thin and slow growth was seen at 20 ^{0}C (Plate 10). Thus *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* showed optimum mycelial growth at 30, 25 to 30 and 25 ^{0}C respectively (Plate 11).

4.6.3. Growth of *Pleurotus* spp. in different pH

The physiological characteristics of isolates *viz., P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were studied at four different pH levels and the results of the study are presented in Table 10 and Plate 12. *P. cystidiosus* (coremial) and *P. cystidiosus* (non-coremial) took the minimum time for completion of petridish at pH 8 (27.5, 5 days) which was significantly different from the other three pH levels. The next best treatments were pH 7 (29.25 days, 6.25 days), 6 (32 days, 7.75 days) and 5 (39.75 days, 26.5 days). Coremial isolate recorded white, thick and fluffy growth with radiating margin at pH 8 whereas, white and thin growth was seen at other pH levels. Non-coremial isolate showed white, thick and cottony growth with concentric zonations at pH 8 and white, thin mycelium with radiating margin at pH 7. *P. opuntiae* took the minimum time for completion of mycelial growth at pH 8 (6.20 days) and 7 (6.40 days) with white, thick, cottony mycelium having radiating margin. The next best treatments were pH 6 (8.20 days) and 5 (8.00 days) which were on par with each other. *P. opuntiae* recorded white, fluffy growth with radiating margin at pH 6 and white, thin growth with radiating margin at pH 5. Thus *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* showed optimum mycelial growth at pH levels 8, 8 and 7 to 8 respectively.

Table 10. Influence of pH on mycelial growth of *Pleurotus* spp.

Sl. No.	pH	Nature of mycelial growth			*Days for complete coverage on petri dish		
		P. cystidiosus coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	*P. opuntiae*
1	5	White, thin, and slow	White, thick restricted	White, thin with radiating margin	39.75[a]	26.50[a]	8.00[a]
2	6	White, thin with radiating margin	White, thin with concentric zonations	White, fluffy with radiating margin	32.00[b]	7.75[b]	8.20[a]
3	7	White, thin with diffused	White, thin, radiating	White, thick, cottony with radiating margin	29.25[c]	6.25[c]	6.40[b]
4	8	White, thick, fluffy with radiating margin	White, thick, cottony with concentric zonations	White, thick, cottony with radiating margin	27.50[d]	5.00[d]	6.20[b]
SE m (±)					2.705	5.072	0.523
CD(0.05)					1.575	1.139	0.568

* Average of five replications
Means followed by similar superscripts are not significantly different at 5 % level

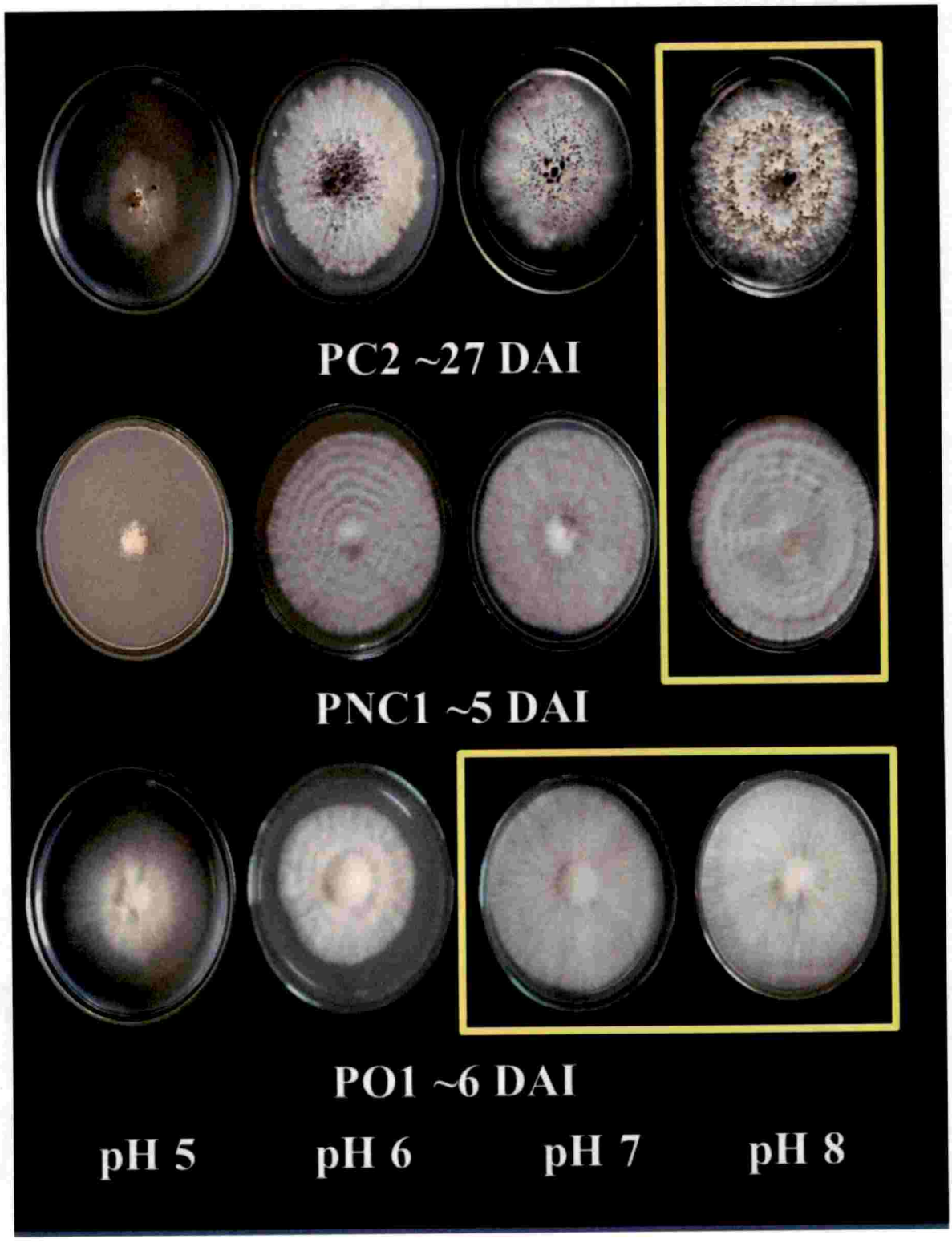


Plate 12. Influence of pH on mycelial growth of *Pleurotus* spp.

4.6.4. Growth of *Pleurotus* spp. in light and dark conditions

The physiological characteristics of fast growing isolates *viz.*, *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* was studied in two different light conditions (Table 11 and Plate 13). Coremial isolate of *P. cystidiosus* and *P. opuntiae* took the minimum time for completion of growth under dark conditions (29.5 days, 6.33 days) with thick and fluffy growth. Dark condition favoured the mycelial growth of *P. cystidiosus* (non-coremial) and took 5.23 days for complete coverage of petri dish.

4.6.5. Amendment studies of *Pleurotus* spp.

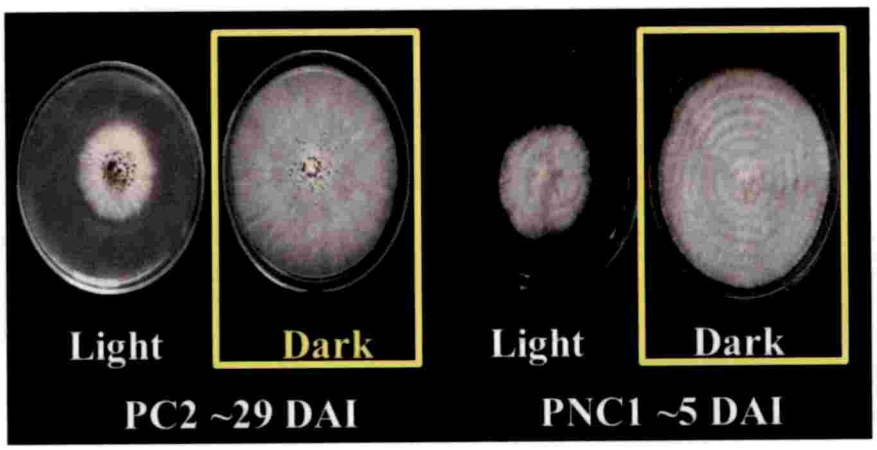
The physiological characteristics of isolates *viz.*, *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were studied in the best medium screened from 4.6.1. (PDPA) amended with the additives *viz.*, thiamine (25, 50 and 75 ppm), yeast (0.5, 0.75 and 1 per cent), iron (0.1, 0.125 and 0.15 per cent), gypsum (2, 4 and 6 per cent) and calcium carbonate (2, 4 and 6 per cent). The results of study are presented in Table 12 and Plate 14.

P. cystidiosus (coremial) took the minimum time for completion of petri dish in PDPA amended with yeast 1 per cent (25.33 days) followed by gypsum 4 per cent (25.67 days), yeast 0.75 per cent (26.00 days) and gypsum 6 per cent (26.00 days). Maximum time for completion of mycelial growth was observed with the amendments *viz.*, thiamine 75 ppm (32.00 days), thiamine 50 ppm (30.00 days) and iron 0.1 per cent (29.67 days). Absence of mycelial growth was observed with iron 0.125 per cent and iron 0.15 per cent. Thick, fluffy growth with radiating margin and maximum coremia was observed with yeast at all three concentrations whereas, thin growth with smooth margin and minimum coremia was observed with thiamine. Very thin, feeble growth with smooth margin was observed with calcium carbonate at three concentrations. Iron 0.1 per cent recorded brown, thin growth with radiating margin. *P. cystidiosus* (non-coremial) took the minimum time for completion of petri dish in PDPA amended with calcium carbonate 4 per cent (3.00 days) followed by calcium carbonate 2 per cent

Table 11. Influence of light and dark conditions on mycelial growth of *Pleurotus* spp.

Sl. No.	Light/ Dark	Nature of mycelial growth			*Days for complete coverage on petri dish		
		P. cystidiosus coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	*P. opuntiae*
1	Light	Thin, sparse	Thin with concentric zonations	Thin, sparse	39.30	11.84	8.14
2	Dark	Thick, fluffy	Thick and cottony with concentric zonations	Thick, fluffy	29.50	5.23	6.33
t (0.05)					2.101**	2.101**	2.101**

*Average of ten replications
** Treatments are significantly different at 5 % level



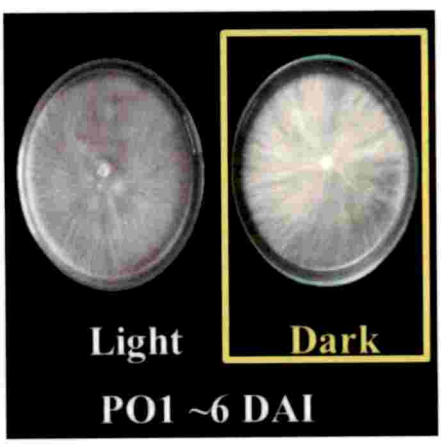


Plate 13. Influence of light and dark conditions on mycelial growth of *Pleurotus* spp.

Table 12. Influence of amendments on mycelial growth of *Pleurotus* spp.

Sl. No.	Amendments	Nature of mycelial growth			*Days for complete coverage on petri dish		
		I	II	III	I	II	III
1	Thiamine 25 ppm	Thin with smooth margin	White, thick, cottony with concentric zonations and radiating margin	White, thin, restricted	29.33^{bc}	6.33^{cd}	10.50^{b}
2	Thiamine 50 ppm				30.00^{b}	7.33^{b}	9.75^{bc}
3	Thiamine 75 ppm				32.00^{a}	9.33^{a}	11.5^{a}
4	Yeast 0.5 %	Thick, fluffy with radiating margin		Thick, fluffy with concentric zonations and radiating margin	29.00^{c}	7.33^{b}	6.5^{ef}
5	Yeast 0.75 %				26.00^{fgh}	6.67^{bc}	6.25^{fg}
6	Yeast 1 %				25.33^{h}	4.00^{g}	5.25^{h}
7	Iron 0.1 %	Brownish, thin with radiating margin			29.67^{bc}	4.33^{fg}	9.5^{c}
8	Iron 0.125 %	No growth		No growth	-	5.00^{ef}	-
9	Iron 0.15 %				-	5.67^{de}	-
10	Gypsum 2 %	Thin, diffused, with smooth margin	White, thick, cottony with radiating margin	Thin, feeble with radiating margin	26.67^{ef}	6.33^{cd}	5.50^{gh}
11	Gypsum 4 %	Thick, cottony, with radiating margin			25.67^{gh}	5.67^{de}	6.25^{fg}
12	Gypsum 6 %	Thin, feeble, with radiating margin			26.00^{fgh}	7.33^{b}	7.25^{e}
13	CC 2 %	Very thin, feeble, with smooth margin	White, very thin, feeble with radiating margin	Very thin, feeble, with radiating margin	27.00^{de}	3.67^{gh}	4.25^{i}
14	CC 4 %				26.33^{efg}	3.00^{h}	8.25^{d}
15	CC 6 %				27.67^{d}	6.00^{cd}	5.50^{gh}
16	Control	White, thin with radiating margin	White, thick cottony with concentric zonations	White, thick with radiating margin	29.67^{bc}	6.00^{cd}	8.25^{d}
SE m (±)					0.546	0.404	0.588
CD (0.05)					0.728	0.791	0.767

*Average of three replications, Means followed by similar superscripts are not significantly different at 5 % level, **Treatments without observation not taken for statistical analysis
CC: Calcium carbonate, I: *P. cystidiosus* coremial, II: *P. cystidiosus* non-coremial, III: *P. opuntiae*

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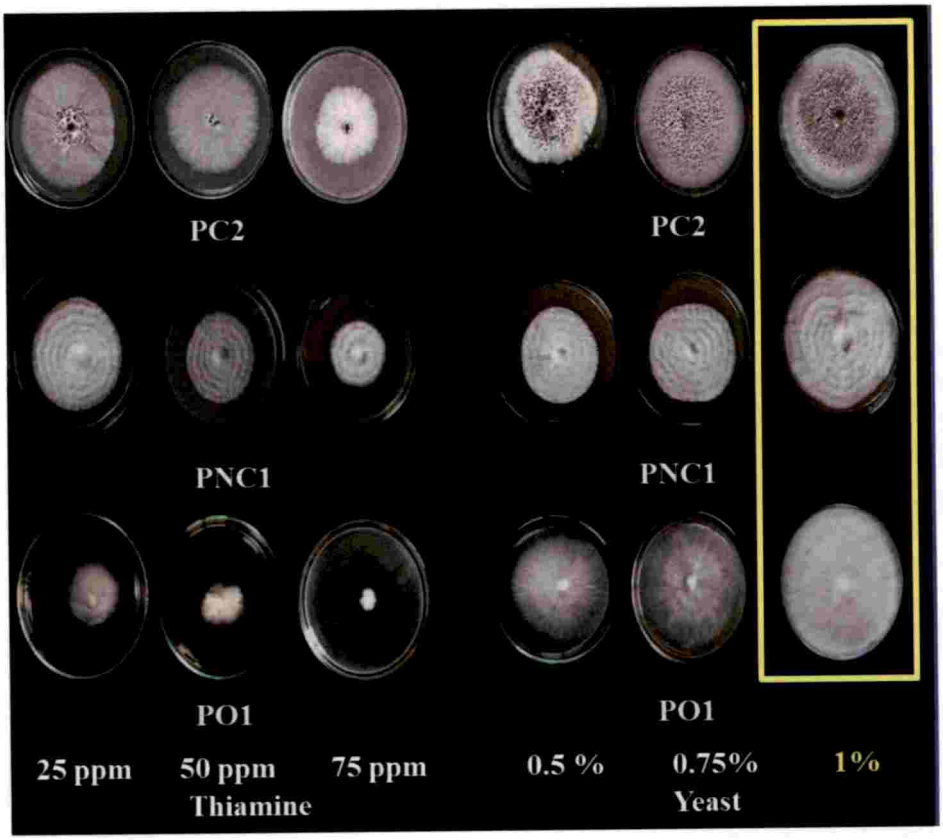


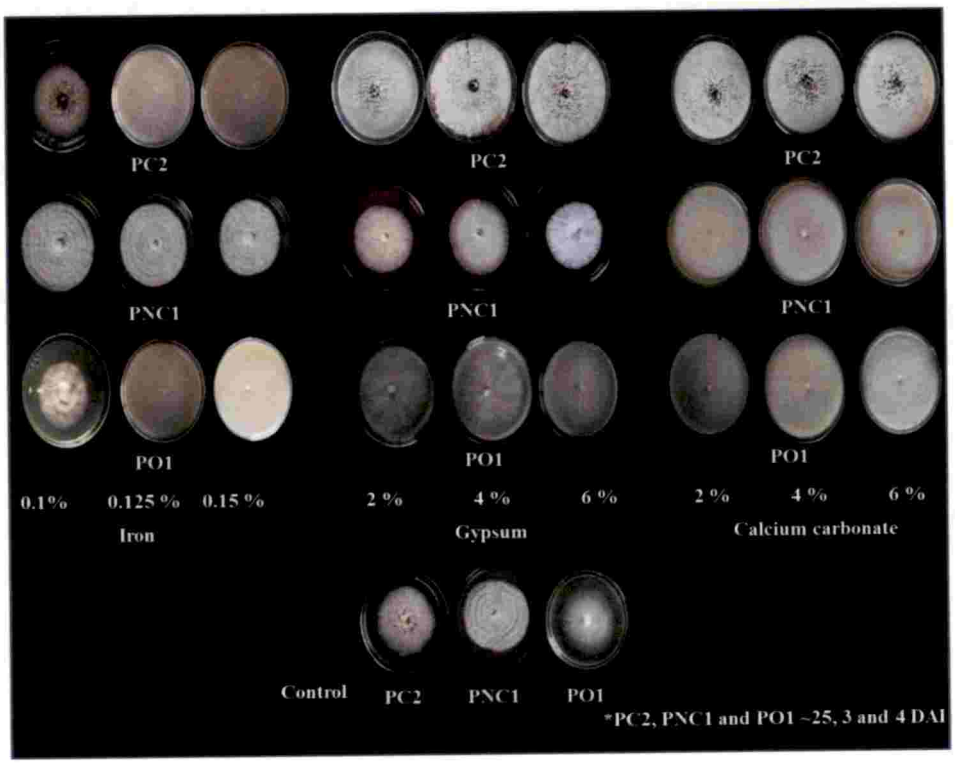


Plate 14. Influence of amendments on mycelial growth of *Pleurotus* spp.

(3.67 days) and yeast 1 per cent (4.00 days). Maximum time for completion of mycelial growth was observed with the amendments *viz*., thiamine 75 ppm (9.33 days), thiamine 50 ppm (7.33 days), yeast 0.5 per cent (7.33 days) and gypsum 6 per cent (7.33 days). However white, thick, cottony growth with concentric zonations and radiating margin was recorded with yeast 1 per cent, hence identified as the best amendment for *P. cystidiosus* (non-coremial). *P. opuntiae* took the minimum time for completion in PDPA amended with calcium carbonate 2 per cent (4.25 days) followed by the amendments *viz*., yeast 1per cent (5.25 days), gypsum 2 per cent (5.50 days) and calcium carbonate 6 per cent (5.50 days). Maximum time for completion of mycelial growth was observed with the amendments *viz.,* thiamine 25 ppm (10.50 days), thiamine 50 ppm (9.75 days) and iron 0.1per cent (9.50 days). Absence of mycelial growth was observed with iron 0.125 per cent and iron 0.15 per cent. Thick, fluffy growth with concentric zonations and radiating margin was recorded with iron 0.1 per cent and yeast at all three concentrations.White, thin and restricted growth was observed with thiamine at three concentrations. Thin and feeble growth with radiating margin was recorded with gypsum and calcium carbonate.Thus, one per cent yeast was screened as the best amendment for the three isolates based on the minimum time taken for complete coverage of petri dish and thickness of mycelial growth.

4.7. SUBMERGED CULTURING OF *PLEUROTUS* SPP.

The submerged culture production of the three isolates *viz., P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* was studied in four different liquid media and observations *viz*., number of pellets 100 ml^{-1}, fresh weight (g) and dry weight of mycelial pellets (g 100 ml^{-1}) obtained for each isolate was determined (Table 13 and Plate 15).

Among the four different liquid media tested for submerged culture production of *P. cystidiosus* (coremial) PDPB was the best giving the highest fresh weight (34.48 g) and biomass (dry weight) (2.82 g) followed by MEB (33.03 g, 2.52 g) and PDB (31.5g, 1.45 g). However maximum number of pellets was

Table 13. Evaluation of submerged culture production of *Pleurotus* spp.

Sl. No.	Broths	*No. of pellets flask^{-1} (100 ml)			*Fresh weight (g)			*Biomass (dry weight, g 100 ml^{-1})		
		P. cystidiosus coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	*P. opuntiae*	*P. cystidiosus* coremial	*P. cystidiosus* non-coremial	*P. opuntiae*
1	PDB	299.50^{b}	273.00^{c}	189.50^{a}	31.50^{c}	34.95^{b}	29.00^{b}	1.45^{c}	1.65^{b}	0.75^{b}
2	MEB	479.25^{a}	278.00^{b}	170.00^{b}	33.03^{b}	28.75^{c}	29.65^{ab}	2.52^{b}	1.25^{c}	0.85^{a}
3	CEB	240.25^{c}	158.25^{d}	99.00^{c}	24.75^{d}	25.90^{d}	16.85^{c}	0.95^{d}	0.48^{d}	0.45^{c}
4	PDPB	152.25^{d}	352.75^{a}	14.00^{d}	34.48^{a}	50.90^{a}	30.17^{a}	2.82^{a}	4.23^{a}	0.87^{a}
SE m (±)		69.116	40.133	39.784	2.151	5.587	3.198	0.441	0.813	0.097
CD (0.05)		2.405	3.064	9.031	0.664	0.745	0.812	0.115	0.128	0.081

PDB: Potato dextrose broth, MEB: Malt extract broth, CEB: Carrot extract broth, PDPB: Potato dextrose peptone broth
*Average of four replications
Means followed by similar superscripts are not significantly different at 5 % level

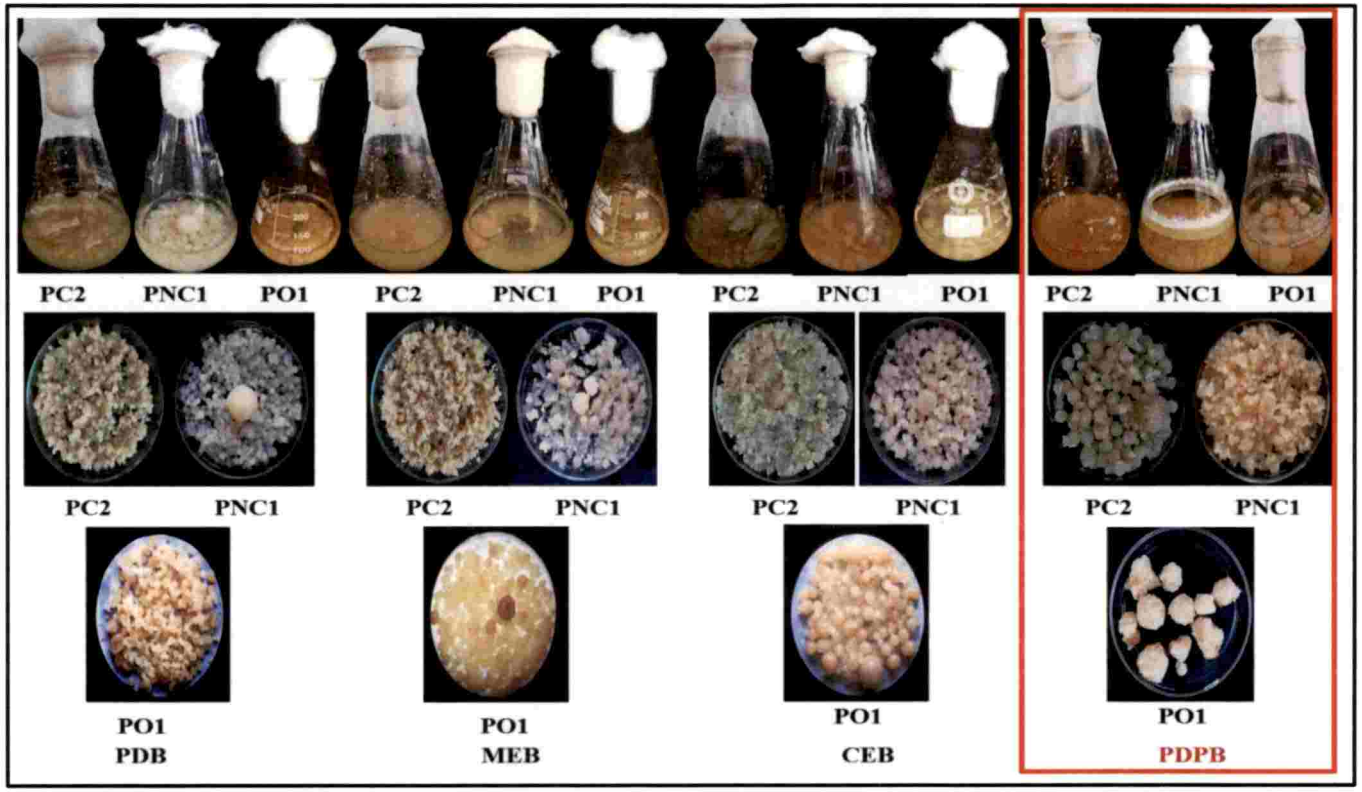


Plate 15. Morphological appearance of *Pleurotus* spp. mycelial pellets in broths

recorded in MEB (479.25) followed by PDB (299.5) and CEB (240.25). Pellets were light yellow to light brown in colour. *P. cystidiosus* (non-coremial) gave the highest number of pellets, fresh weight and biomass (dry weight) with PDPB (352.75, 50.9 g, 4.23 g). Pellets were light cream to light brown in colour. *P. opuntiae* gave the highest fresh weight and biomass with PDPB (30.17g, 0.87 g) followed by MEB (29.65g, 0.85 g) and PDB (29.00g, 0.75 g). Maximum number of pellets was seen with PDB (189.50) followed by MEB (170) and CEB (99.00). Pellets were light cream to light brown in colour.

4.8. COREMIAL CHARACTERS OF *P. CYSTIDIOSUS*

Coremial isolate of *P. cystidiosus* was characterised by the formation of specialised structures named coremia representing its anamorphic stage- *Antromycopsis macrocarpa* (Plate 16). White mycelium started radiating from the fresh mycelial bit kept on the medium from second day of inoculation. From the fifth day, small protuberances started arising on the entire mycelial tissue simultaneously. Protuberance developed in to white stalk with a tiny watery droplet at its tip, which later turned blackish colour on the white stalk after 24 h. As the mycelium started maturing and radiating out from the bit, black droplets were also seen radiating outwards from the centre. Microscopic observation of the coremial liquid revealed elliptical and round spores of sizes 16.31 µm x 7.48 µm and 8.06 µm x 8.49µm respectively (Plate 17). The spore-laden black droplets did not pose a contamination threat to other cultures in the laboratory. If petri dishes are not handled carefully, the droplets will streak across the media freeing the conidial spores. The imperfect/coremial stage provides the cultivator with the advantage of simultaneous asexual spore mass inoculation enabling faster colonisation.

4.8.1. Isolation and characterization of melanin from *P. cystidiosus*

The melanin pigment from coremial liquid of *P. cystidiosus* was extracted with 1 *M* sodium hydroxide as per the standard procedure described by Gadd

(1982). The partially purified pigment answered positively to the chemical tests (Table 14 and Plate 18) which are diagnostic for fungal melanin as described by Thomas (1955). The extracted melanin was brownish-black in colour, insoluble in water and organic solvents, soluble in 1 *M* KOH for 2 h at 100 ^{0}C, precipitated in 3 *N* HCl, gave reddish brown precipitate with ferric chloride one per cent (reaction for polyphenols), precipitated with ammoniacal silver nitrate solution and decolorized with hydrogen peroxide 3 per cent (oxidising agent). The nature of the pigment was further confirmed by its spectral properties. Its UV spectrum was typical of the absorption profile of melanin. The absorption spectra showed characteristic absorption peak in the UV regions around 250 nm.

4.8.2. Antioxidant and antiproliferative activity of melanin from *P. cystidiosus*

Antioxidant activity of melanin was analyzed by DPPH free radical scavenging assay. Melanin pigment from *P. cystidiosus* showed high antioxidant and free radical scavenging activities. When the concentration of extracellular melanin produced from *P. cystidiosus*, was increased from 200 to 800 µg the DPPH radical scavenging activity or per cent inhibition also increased from 30.90 to 35.40 per cent (Table 15). IC 50 value was calculated from log dose inhibition curve and recorded as 1000 µg ml^{-1}.

The MTT or *in-vitro* cell proliferation assay was used to study the invitro antiproliferative activity of extracted melanin against colon cancer cell line (HeLa). The viability of colon cancer cells treated with melanin extract reduced in a concentration dependent manner (Table 16). Higher extract concentrations exhibited stronger anticancer activity. Dried extract of mushroom at concentration of 100 µg ml^{-1} recorded the highest anti-proliferative effect, with least absorbance (0.594) at 540 nm and minimum cell viability (45.99 per cent). The IC-50 value was recorded as 78.29 µg ml^{-1} *i.e.* concentration of extract needed for the 50 per cent of cell death. Results indicated that the extracted melanin had significant anti-proliferative activity against colon cancer cell lines.

Table 14. Identification parameters for melanin extracted from *P. cystidiosus* (coremial)

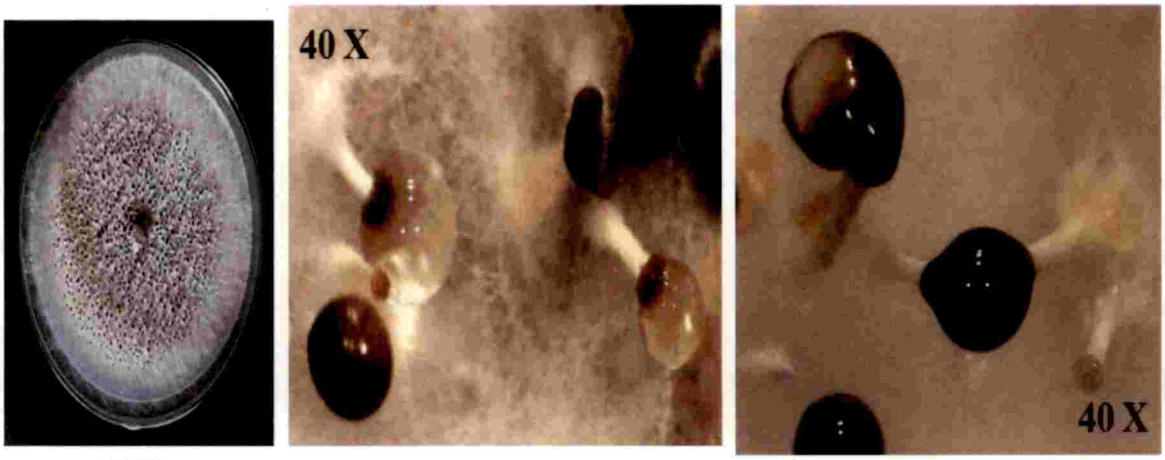
Sl No.	Test	Result
1.	Colour	brownish-black
2.	Solubility in water	insoluble
3.	Solubility in organic solvents	insoluble
4.	Solubility in 1 *M* potassium hydroxide (at 100^0 C, 2 h)	soluble
5.	Precipitation in 3 *N* Hydrochloric acid	precipitated
6.	Reaction for poly phenols (1% Ferric chloride)	precipitated
7.	Reaction with ammoniacal silver nitrate solution	precipitated
8.	Reaction with oxidizing agent (3% Hydrogen peroxide)	decolourised

Table 15. DPPH radical scavenging activity of the extracted melanin at various doses

Sample	*Absorbance at 516 nm	*Per cent inhibition
Control at 0 min	0.62^{a}	0^{e}
Control at 15 min	0.47^{d}	0^{e}
T1 (200µg)	0.48^{b}	30.90^{d}
T2 (400 µg)	0.47^{c}	32.40^{c}
T3 (600 µg)	0.46^{e}	34.76^{b}
T4 (800 µg)	0.46^{f}	35.40^{a}
SE m (±)	0.025	7.065
CD (0.05)	0.001	0.000

IC 50 value-1000µg/ml

*Average of three replications, Means followed by similar superscripts are not significantly different at 5 % level



PC2
showing coremia

Coremia under stereomicroscope

Plate 16. Anamorphic stage *of P. cystidiosus* (coremial)

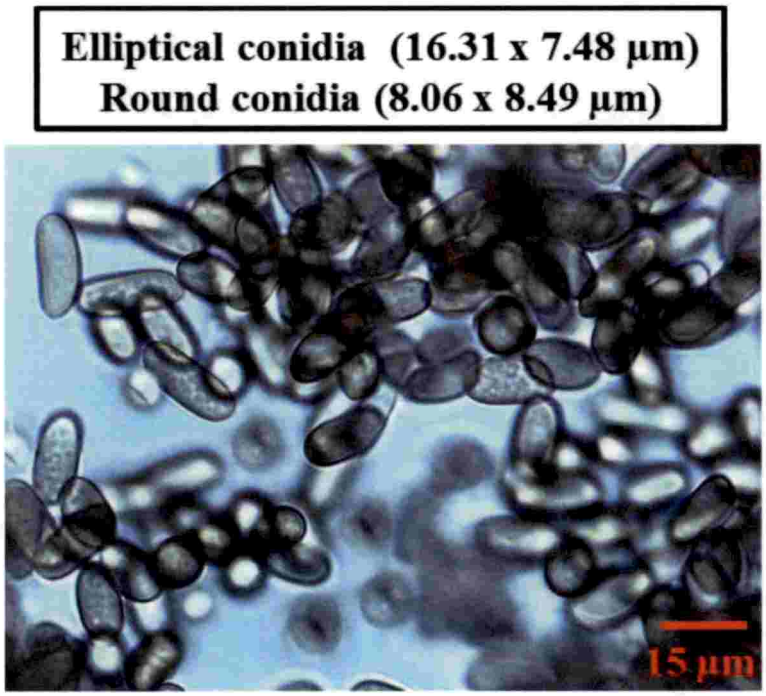


Plate 17. Conidia of coremia

PC2 in PD broth
(10 WAI)

Melanin dried at 20^{0}C, 10 h
: 255.56 mg/l

Plate 18. Isolation of melanin produced by *P. cystidiosus*

Table 16. Invitro anti-proliferative activity of melanin against colon cancer cell line (HeLa)

Parameters	Sample concentration (µg ml^{-1})					IC 50 value (µg ml^{-1})
	6.25	12.50	25.00	50.00	100.00	
*Absorbance (540 nm)	1.091	0.929	0.837	0.700	0.594	78.29
*Per cent viability (%)	84.47	71.95	64.81	54.16	45.99	

*Average of five replications

4.9. EVALUATION OF DIFFERENT SUBSTRATES FOR SPAWN PRODUCTION OF PLEUROTUS SPP.

Three substrates *viz*., paddy grain, sorghum and rubber wood sawdust were evaluated for production of spawn of isolates *viz., P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* on the basis of days for spawn run, nature of mycelial growth, presence of contaminants and shelf life. Spawns prepared from the substrates *viz.*, paddy grains, sorghum and rubber sawdust were separately amended with additives at different concentrations *viz*., thiamine (25, 50 and 75 ppm), yeast extract (0.5, 0.75 and 1 per cent) and iron in the form of ferrous sulphate (0.25, 0.5 and 0.75 per cent).

P. cystidiosus (coremial) took the minimum time for spawn run in paddy grains when amended with yeast 1 per cent (23.07 days) followed by yeast 0.75 per cent (23.32 days), thaimine 25 ppm (23.32 days) and yeast 0.5 per cent (23.63 days). Maximum time for spawn run was recorded with iron 0.75 per cent (29.07 days) followed by iron 0.5 per cent (27.35 days) and thiamine 75 ppm (27.30 days). Yeast at three concentrations, iron 0.25 per cent and iron 0.5 per cent resulted in thicker and fluffy mycelial growth whereas, poor mycelial growth was recorded with thiamine 50 ppm, thiamine 75 ppm and iron 0.75 per cent. Absence of contamination was recorded with thiamine 25 ppm, iron 0.25 per cent and yeast at all three concentrations whereas, maximum contamination was recorded with iron 0.75 per cent and thiamine 75 ppm (60.00 per cent). Maximum shelf life was recorded with yeast 1 per cent (85.62 days) followed by yeast 0.75 per cent (85.30 days) and yeast 0.5 per cent (84.30 days) (Table 18 and plate 17). *P. cystidiosus* (non-coremial) took the minimum time for spawn run in paddy grains amended with yeast 1 per cent (5.63 days) followed by yeast 0.75 per cent (6.17 days) and iron 0.25 per cent (6.31 days) with thicker and fluffy growth. Maximum time for spawn run was recorded with iron 0.75 per cent (12.63 days) followed by thiamine 75 ppm (12.33 days) and thiamine 50 ppm (11.30 days) with poor growth. Amendments *viz.*, yeast 0.75 per cent, yeast 1 per cent and iron 0.25 per

Table 17. Evaluation of amended paddy grains for spawn production of *Pleurotus* spp.

Sl. No.	Treatments	*Days for spawn run			Nature of growth			Contaminants			*Per cent spawns contaminated			*Shelf life (days)		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	Thiamine 25 ppm	23.32^{f}	8.35^{e}	16.32^{e}	+++	++	+++	-	*A*	*T.*	-	53.3	40	82.32^{e}	75.33^{f}	57.30^{e}
2	Thiamine 50 ppm	26.32^{c}	11.30^{c}	17.07^{d}	++	++	+++	*A*	*M*	*T, B*	33.3	60	40	82.07^{f}	72.37^{h}	56.41^{f0}
3	Thiamine 75 ppm	27.30^{b}	12.33^{b}	32.63^{a}	++	++	++	*T*	*T, A*	*T, A, R*	60.0	60	60	81.30^{g}	71.35^{i}	43.30^{i}
4	Yeast 0.5 %	23.63^{e}	7.33^{f}	12.63^{g}	++++	+++	++++	-	*T*	*T*	-	33.3	40	84.30^{c}	79.30^{c}	62.20^{c}
5	Yeast 0.75 %	23.32^{f}	6.17^{h}	12.32^{h}	++++	++++	++++	-	-	*T*	-	-	40	85.30^{b}	80.30^{b}	62.60^{b}
6	Yeast 1 %	23.07^{g}	5.63^{i}	11.17^{i}	++++	++++	++++	-	-	-	-	-	-	85.60^{a}	81.52^{a}	63.60^{a}
7	Iron 0.25 %	25.35^{d}	6.31^{h}	15.17^{f}	++++	++++	+++	-	-	*T*	-	-	40	80.28^{h}	75.65^{e}	56.30^{f}
8	Iron 0.50 %	27.35^{b}	9.07^{d}	28.32^{c}	++++	++	++	*P*	*T, R*	*T, A*	33.3	60	53	79.35^{i}	74.32^{g}	47.30^{g}
9	Iron 0.75 %	29.07^{a}	12.63^{a}	30.30^{b}	++	++	++	*P, T*	*R*	*T, M, P*	60.0	60	60	79.07^{j}	71.33^{i}	45.30^{h}
10	Control	23.07^{g}	7.07^{g}	16.17^{e}	+++	+++	+++	*T*	*T, A*	*T*	33.3	33.3	40	82.63^{d}	76.42^{d}	58.30^{d}
SE m (±)		0.698	0.828	2.538										0.733	1.158	2.340
CD (0.05)		0.114	0.184	0.278										0.090	0.175	0.146

*Average of three replications, each replication denotes 5 spawns, Means followed by similar superscripts not significantly different at 5% level
++++: Thicker and fluffy growth; +++:Thick growth; ++: Poor growth, *A*: *Aspergillus* sp, *T*: *Trichoderma* sp, *B*: *Bacillus* sp, *R*: *Rhizopus* sp, *M*: *Mucor* sp., *P: Penicillium* sp., Control: paddy grains alone
I: *P. cystidiosus* coremial, II: *P. cystidiosus* non-coremial, III: *P. opuntiae*

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Table 18. Evaluation of amended sorghum for spawn production of *Pleurotus* spp.

Sl. No.	Treatments	*Days for spawn run			Nature of growth			Contaminants			*Per cent spawns contaminated			*Shelf life (days)		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	Thiamine 25 ppm	18.62^{d}	10.33^{e}	15.00^{d}	+++	+++	+++	-	*T*	*T*	-	33.3	40.0	53.00^{e}	48.37^{e}	35.00^{e}
2	Thiamine 50 ppm	19.17^{c}	14.63^{b}	15.67^{c}	++	++	+++	*T*	*A*	*T*	33.3	53.3	46.6	52.35^{f}	45.35^{h}	34.63^{f}
3	Thiamine 75 ppm	19.33^{c}	17.07^{a}	16.67^{a}	++	++	++	*T*	*T, B*	*T, B*	33.3	53.3	46.6	52.00^{g}	42.32^{i}	34.30^{g}
4	Yeast 0.5 %	17.62^{f}	8.32^{g}	11.00^{g}	++++	+++	++++	-	*T*	*T*	-	33.3	40.0	55.31^{c}	51.33^{c}	36.30^{c}
5	Yeast 0.75%	17.32^{g}	6.63^{h}	10.66^{h}	++++	++++	++++	-	-	-	-	-	-	56.30^{b}	53.33^{b}	38.30^{b}
6	Yeast 1 %	17.32^{g}	6.30^{i}	9.17^{i}	++++	++++	++++	-	-	-	-	-	-	56.60^{a}	54.32^{a}	38.60^{a}
7	Iron 0.25 %	19.17^{c}	6.62^{h}	11.17^{fg}	++	++++	+++	*T*	-	*T*	33.3	-	40.0	50.30^{h}	48.32^{e}	35.30^{d}
8	Iron 0.50 %	19.63^{b}	12.27^{d}	13.34^{e}	++	++	+++	*A*	*P*	*T, A*	40.0	53.3	40.0	50.07^{i}	47.32^{f}	34.30^{g}
9	Iron 0.75 %	20.32^{a}	13.63^{c}	16.33^{b}	++	++	++	*A*	*A*	*T, A, P*	60.0	60.0	60.0	49.30^{j}	46.32^{g}	33.60^{h}
10	Control	18.35^{e}	10.07^{f}	11.31^{f}	+++	+++	+++	-	*T*	*T, A*	-	33.3	40.0	53.30^{d}	49.32^{d}	35.30^{d}
SE m (±)		0.324	1.182	0.857										0.819	1.155	0.535
CD(0.05)		0.229	0.103	0.227										0.105	0.069	0.065

*Average of three replications, each replication denotes 5 spawns, ++++ :Thicker and fluffy growth; +++ :Thick growth; ++ :Poor growth, Control: sorghum alone

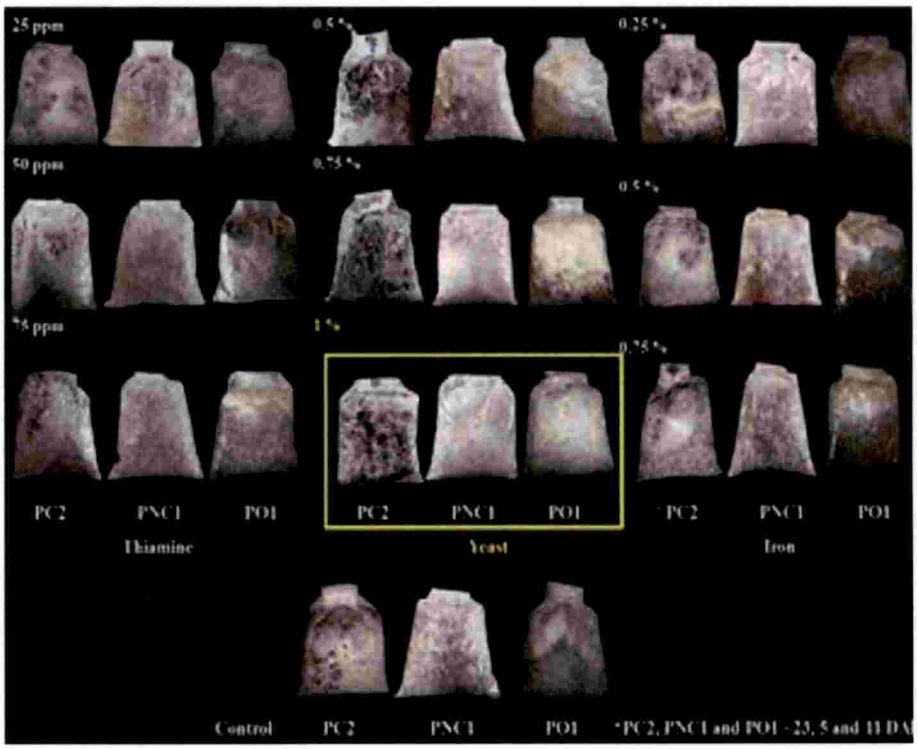


Plate 19. Evaluation of amended paddy grains for spawn production of *Pleurotus* spp.

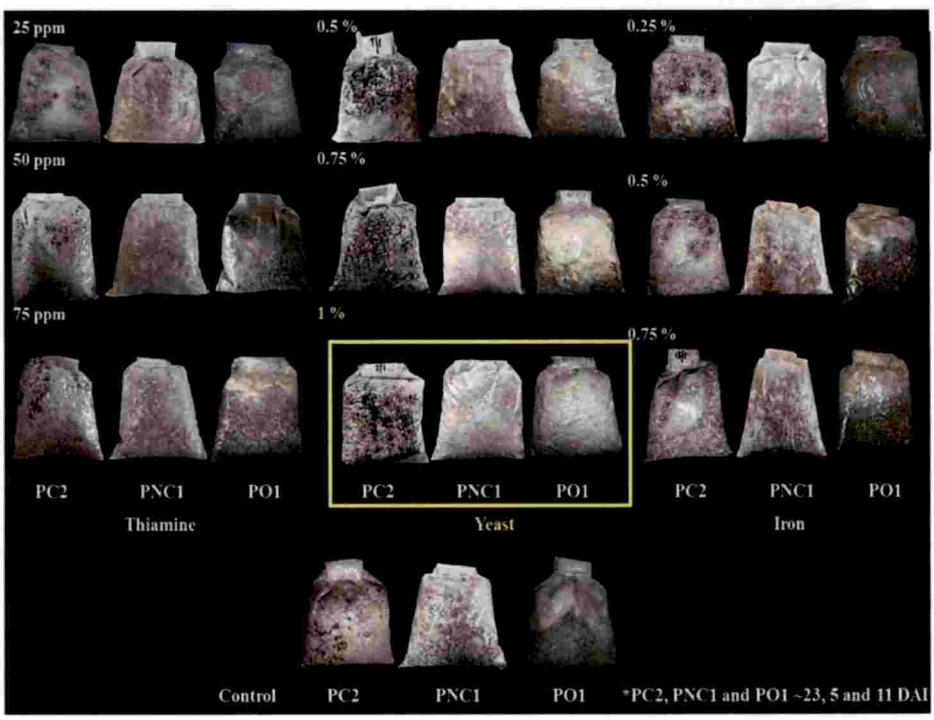


Plate 20. Evaluation of amended sorghum for spawn production of *Pleurotus* spp.

cent recorded absence of contamination followed by yeast 0.5 per cent and paddy grain alone (33.33 per cent each). Maximum shelf life was recorded with yeast 1 per cent (81.58 days) followed by yeast 0.75 per cent (80.35 days) and yeast 0.5 per cent (79.30 days). *P. opuntiae* took minimum time for completion of spawn run in paddy grains with yeast 1 per cent (11.17 days) followed by yeast 0.75 per cent (12.32 days) and yeast 0.5 per cent (12.63 days) with thicker and fluffy growth. Thiamine 75 ppm took the maximum time for spawn run (32.63 days) followed by iron 0.75 per cent (30.30 days) and iron 0.5 per cent (28.32 days) with poor mycelial growth. Absence of contamination was recorded with yeast 1 per cent. Maximum contamination was observed with iron 0.75 per cent and thiamine 75 ppm (60.00 per cent). Maximum shelf life was recorded with yeast 1 per cent (63.67 days), yeast 0.75 per cent (62.63 days) and yeast 0.5 per cent (62.20 days) (Table 17 and Plate 19).

P. cystidiosus (coremial) took the minimum time for spawn run in sorghum grains with yeast 0.75 per cent and yeast 1 per cent (17.32 days) followed by yeast 0.5 per cent (17.62 days) with thicker and fluffy growth. Maximum time for mycelial growth was recorded with the amendments *viz.*, iron 0.75 per cent (20.32 days), iron 0.50 per cent (19.63 days) and iron 0.25 per cent (19.17 days). Thiamine 25 ppm, yeast at all three concentrations and sorghum alone recorded absence of contamination whereas, iron 0.75 per cent recorded maximum contamination (60.00 per cent). Maximum shelf life was seen with yeast 1 per cent (56.67 days) followed by yeast 0.75 per cent (56.32 days) and yeast 0.5 per cent (55.35 days). *P. cystidiosus* (non-coremial) took the minimum time for spawn run in sorghum grains amended with yeast 1 per cent (6.30 days) followed by iron 0.25 per cent (6.62 days) and yeast 0.75 per cent (6.63 days), with thicker and fluffy growth. Maximum time for spawn run was seen with thiamine 75 ppm(17.07 days), thiamine 50 ppm (14.63 days) and iron 0.75 per cent (13.63 days), with poor mycelial growth. Absence of contamination was recorded with yeast 0.75 per cent, yeast 1 per cent and iron 0.25 per cent whereas, maximum contamination was recorded with iron 0.75 per cent (60.00 per cent).

Maximum shelf life was recorded with yeast 1 per cent (54.32 days) followed by yeast 0.75 per cent (53.33 days) and yeast 0.5 per cent (51.33 days) (Table 19 and Plate 18). *P. opuntiae* took minimum period for completing spawn run in sorghum grains amended with 1 per cent yeast (9.17 days) followed by yeast 0.75 per cent (10.66 days) and yeast 0.5 per cent (11.00 days), showing thicker and fluffy mycelial growth. Maximum time for spawn run was recorded with thiamine 75 ppm (16.67 days) followed by iron 0.75 per cent (16.33 days) and thiamine 50 ppm (15.67 days). Contaminants was not observed in sorghum spawns amended with yeast 0.75 per cent and yeast 1 per cent, whereas maximum contamination was recorded with iron 0.75 per cent (60 per cent). Maximum shelf life was observed with the amendment yeast 1 per cent (38.67 days) followed by yeast 0.75 per cent (38.32 days) and yeast 0.5 per cent (36.30 days) (Table 18 and Plate 20).

P. cystidiosus (coremial) took the minimum time for spawn run in rubber wood sawdust, with 1 per cent yeast (23.07 days) followed by 0.75 per cent yeast (23.63 days) and sawdust alone (24.32 days). Maximum time for spawn run was recorded with thiamine 75 ppm (32.32 days) followed by thiamine 50 ppm (29.17 days) and iron 0.75 per cent (27.63 days). Thiamine at three concentration gave thick mycelial growth whereas, yeast at three concentrations gave a thicker and fluffy growth. Absence of contamination was recorded with yeast at all three concentrations and iron 0.25 per cent whereas, all the other treatments recorded contamination of 33.33 per cent. Maximum shelf life was recorded with yeast 1 per cent (114.27 days), yeast 0.75 per cent (113.30 days) and yeast 0.5 per cent (112.32 days) (Table 20 and Plate 19). *P. cystidiosus* (non-coremial) took the minimum time for spawn run in rubber wood sawdust amended with yeast 1 per cent (8.33 days) followed by yeast 0.75 per cent (8.62 days) and iron 0.25 per cent (8.62 days) having thicker and fluffy growth. Maximum time for spawn run was recorded with thiamine 75 ppm (15.35 days), thiamine 50 ppm (14.27 days) and thiamine 25 ppm (12.62 days). Absence of contamination was recorded with yeast at all three concentrations, iron 0.25 per cent, 0.5 per cent iron and sawdust alone.

Table 19. Evaluation of amended rubber wood sawdust for spawn production of *Pleurotus* spp.

Sl. No.	Treatments	*Days for spawn run			Nature of mycelial growth			Contaminants			*Per cent spawns contaminated			*Shelf life (days)		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	Thiamine 25 ppm	24.65^{e}	12.62^{c}	19.07^{c}	+++	+++	+++	*T*	*T*	*T*	33.3	33.3	33.3	110.30^{e}	103.30^{g}	69.35^{h}
2	Thiamine 50 ppm	29.17^{b}	14.27^{b}	22.35^{b}	+++	+++	+++	*T*	*T*	*T*	33.3	33.3	33.3	109.60^{f}	101.30^{h}	66.33^{i}
3	Thiamine 75 ppm	32.32^{a}	15.35^{a}	23.10^{a}	+++	++	++	*T*	*A*	*T, A*	33.3	33.3	40.0	109.30^{g}	100.30^{j}	65.32^{j}
4	Yeast 0.5%	27.33^{d}	11.35^{e}	18.13^{e}	++++	++++	++++	-	-	*T*	-	-	33.3	112.30^{c}	104.10^{e}	76.32^{c}
5	Yeast 0.75%	23.63^{g}	8.62^{g}	17.63^{f}	++++	++++	++++	-	-	*T*	-	-	33.3	113.30^{b}	107.30^{b}	78.32^{b}
6	Yeast 1%	23.07^{h}	8.33^{h}	17.07^{g}	++++	++++	++++	-	-	-	-	-	-	114.20^{a}	108.30^{a}	78.67^{a}
7	Iron 0.25%	24.63^{e}	8.62^{g}	16.67^{h}	+++	++++	+++	-	-	*T*	-	-	33.3	106.30^{h}	104.30^{d}	73.32^{e}
8	Iron 0.5%	27.32^{d}	10.63^{f}	17.07^{g}	++	++++	++	*T*	-	*T, P*	33.3	-	40.0	105.30^{j}	103.60^{f}	72.32^{f}
9	Iron 0.75%	27.63^{c}	12.32^{d}	18.32^{d}	++	++++	++	*A*	*T*	*T, R*	33.3	33.3	40.0	105.60^{i}	101.10^{i}	71.35^{g}
10	Control	24.32^{f}	11.35^{e}	16.67^{h}	+++	++	+++	*T*	-	*T*	33.3	-	33.3	110.60^{d}	104.60^{c}	74.32^{d}
SE m (±)		0.914	0.757	0.730										0.995	0.811	1.458
CD (0.05)		0.175	0.147	0.168										0.055	0.111	0.061

*Average of three replications, each replication denotes 5 spawns, ++++ : Thicker and fluffy growth; +++ : Thick growth; ++ : Poor growth, Control: Rubber wood sawdust alone

Plate 21. Evaluation of amended rubber wood sawdust for spawn production of *Pleurotus* spp.

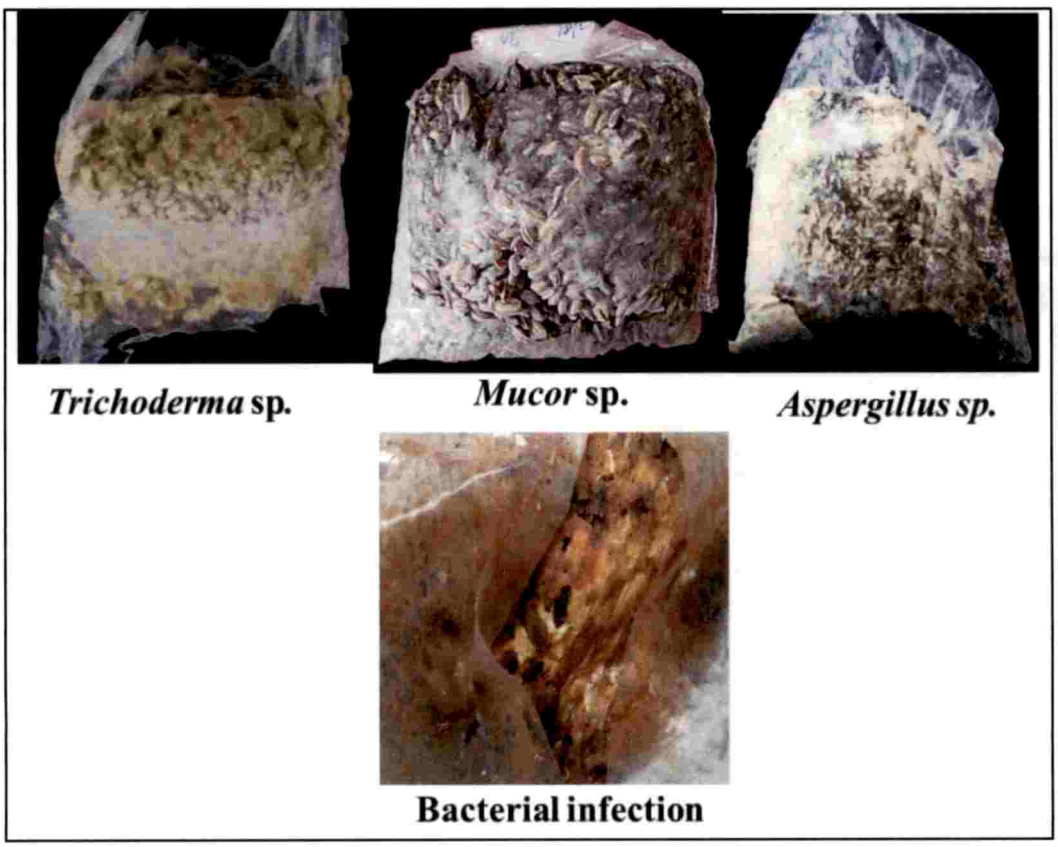


Plate 22. Contaminants observed in spawns of *Pleurotus* spp.

Maximum shelf life was recorded with yeast 1 per cent (108.32 days) followed by yeast 0.75 per cent (107.35 days) and sawdust alone (104.62 days). *P. opuntiae* took the minimum time for completion of spawn run in rubber wood sawdust amended with iron 0.25 per cent (16.67 days) which was on par with rubber wood sawdust alone (16.67 days) having thick mycelial growth. None of the amendments could improve the time taken for spawn run in rubber wood sawdust. However, yeast at all three concentrations resulted in thicker and fluffy growth, compared to other treatments. Absence of contamination was noticed when yeast 1 per cent was used as amendment. Maximum shelf life was recorded with yeast 1 per cent (78.67 days) followed by yeast 0.75 per cent (78.32 days) and yeast 0.5 per cent (76.32 days) (Table 19 and Plate 21). Among the different substrates tried for spawn production of *P. opuntiae*, all the substrates were able to produce primordia as well fruiting bodies in the spawn itself. *Trichoderma* spp. and *Aspergillus* spp. were the main fungal contaminants observed on paddy grains, sorghum and rubber wood sawdust of the three *Pleurotus* isolates (Plate 22).

4.10. CULTIVATION

Multilocation trials with the fast growing isolates of *P. cystidisous* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were carried out in Idukki and Vellayani. Results of the study are presented in Table 20 and Plate 23.

P. cystidiosus (coremial) produced fruiting body during October-January of 2016 (23.68/31.99 ^{0}C, 76.16/92.28 per cent RH) in Vellayani with 33 per cent BE. Hence, *P. cystidiosus* (coremial) was not used for further amendment studies, due to its erratic nature of production. However *P. cystidiosus* non-coremial and *P. opuntiae* recorded high biological efficiencies in Idukki and Vellayani, respectively (Plate 24 and plate 25). **Hence, amendment studies were carried out with the two screened isolates.** Different substrates *viz.*, paddy straw, sawdust of hardwood trees (rubber wood sawdust) and coirpith (degraded, washed and sterilised coirpith) were evaluated for screening the most suitable substrate for fruiting body production of *P. cystidiosus* (non-coremial) and *P. opuntiae*. Nitrogen supplements *viz.,* wheat bran (2 and 4 per cent), rice bran (2 and 4 per

Table 20. Multilocation trials done with the isolates of *Pleurotus* spp.

Sl. No.	Locations	PC2	PNC1	PO1
1	Trivandrum	++	+	++++
2	Idukki	-	++++	+++

*-: No sporocarps, +: immature sporocarps, ++: low B.E, +++: medium BE, ++++: high BE

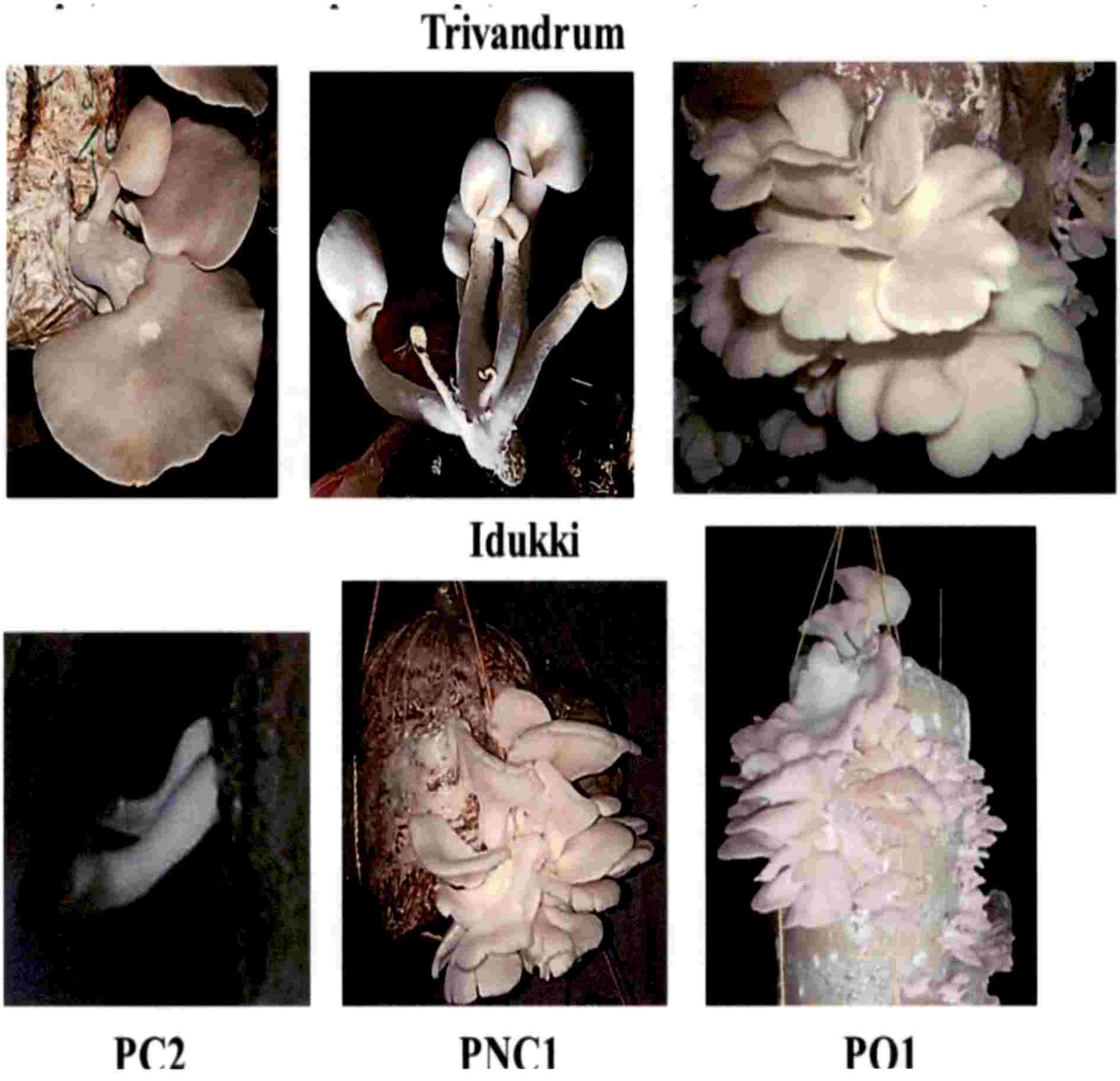


Plate 23. Multilocation trials with isolates

Plate 24. Cultivation trial with *P. opuntiae* in Trivandrum

Plate 25. Farmer's field trial with *P. cystidiosus* (non-coremial) in Idukki

cent), neem cake (2 and 4 per cent) and chemical sprays *viz.*, urea (0.5 and 1 per cent), gypsum (1 and 2 per cent), 1 *M* KH_2PO_4 (2 and 2.5 per cent) and gibberellic acid (GA) (10 and 20 ppm) were used as amendments for the different substrates *viz.*, paddy straw, rubber wood sawdust and coirpith in polybag method of cultivation.

4.10.1. Evaluation of amendments for mushroom production of *P. cystidiosus* (non-coremial)

When *P. cystidiosus* (non-coremial) was cultivated in paddy straw minimum time for spawn run, pinhead formation and first harvest was recorded with 1*M* KH_2PO_4 2 per cent spray (35.62, 40.61 and 44.47 days) followed by 1 *M* KH_2PO_4 2.5 per cent spray (35.48, 40.63 and 44.62 days) and urea 1 per cent spray (35.62, 40.62 and 44.62 days). Total yield per bed from three harvests, BE and crop period were noted to be the highest with wheat bran 4 per cent (2510.32 g, 125.52 per cent, 124.32 days), followed by 1 *M* KH_2PO_4 2.5 per cent spray (2495.31 g, 124.77 per cent, 123.62 days) and wheat bran 2 per cent (2361.32 g, 118.07 per cent, 123.42 days). Average weight of sporocarps was recorded to be maximum with wheat bran 4 per cent (23.17 g) followed by 1 *M* KH_2PO_4 2.5 per cent spray (18.12 g), gypsum 1 per cent spray (17.89 g) and rice bran 4 per cent (17.83 g). Maximum number of sporocarps was recorded with urea 0.5 per cent spray (253.42) followed by 1 *M* KH_2PO4 2 per cent spray (244.33) and wheat bran 2 per cent (186.37) (Table 21 and Plate 26).

When *P. cystidiosus* (non-coremial) was cultivated in rubber wood sawdust minimum time for spawn run, pinhead formation and first harvest was recorded with 1 *M* KH_2PO_4 2.5 per cent spray (45.15, 51.30 and 56 days), followed by 1 *M* KH_2PO_4 2 per cent (45.25, 51.20 and 56 days) and urea 0.5 per cent (45.22, 51.63 and 56.30 days). Total yield from three harvests, BE and crop period was recorded highest for 1 *M* KH_2PO4 2.5 per cent spray (3855.12 g, 192.76 per cent, 145.63 days) followed by wheat bran 4 per cent (3815.41g,

Table 21. Influence of amended paddy straw on yield parameters of *Pleurotus* spp.

Sl. No.	Treatments	Days for complete spawn run*		Days for pinhead formation*		Days for first harvest*		Total yield per bed from three harvests (g)*		Total crop period (days)*		Average weight of sporocarp (g)*		Number of sporocarps*		Biological efficiency (%)*		BC ratio	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
1	2% WB	39.29^{b}	9.29^{e}	44.40^{d}	10.29^{h}	47.62^{d}	12.67^{g}	2361.32^{c}	669.67^{g}	123.42^{c}	39.31^{e}	12.72^{f}	4.17^{c}	186.37^{c}	160.31^{k}	118.07	66.96	8	2.23
2	4% WB	39.57^{a}	9.61^{d}	44.62^{c}	10.67^{g}	48.63^{c}	12.66^{g}	2510.32^{a}	806.05^{a}	124.32^{a}	42.00^{a}	23.17^{a}	4.42^{b}	140.33^{h}	181.63^{h}	125.52	80.60	8.37	2.69
3	2% RB	37.63^{e}	11.00^{c}	43.32^{f}	12.01^{d}	47.00^{f}	15.36^{d}	1220.12^{l}	662.66^{h}	120.32^{f}	36.32^{h}	15.37^{e}	4.22^{c}	65.33^{op}	155.31^{l}	61.01	66.26	4.07	2.21
4	4% RB	38.31^{d}	11.30^{b}	43.62^{e}	12.35^{c}	47.63^{d}	15.61^{c}	1611.42^{g}	722.66^{f}	121.33^{e}	38.32^{g}	17.83^{c}	3.31^{e}	90.33^{l}	216.31^{e}	80.57	72.26	5.37	2.41
5	2% NC	38.63^{c}	12.57^{a}	43.28^{f}	13.30^{b}	47.27^{e}	16.63^{b}	1202.38^{m}	561.65^{k}	119.33^{h}	39.00^{f}	15.91^{d}	3.12^{f}	76.33^{n}	180.63^{i}	60.12	56.16	4.01	1.87
6	4% NC	38.22^{d}	12.61^{a}	42.62^{g}	13.67^{a}	46.63^{g}	17.25^{a}	1660.32^{f}	760.66^{e}	119.67^{g}	39.32^{e}	12.75^{f}	4.70^{a}	130.33^{i}	161.31^{j}	83.02	76.06	5.53	2.53
7	0.5% U	36.33^{g}	8.61^{f}	41.30^{i}	9.63^{jk}	45.31^{i}	12.62^{g}	1320.35^{j}	578.52^{i}	112.33^{l}	35.65^{j}	5.37^{k}	4.41^{b}	253.42^{a}	130.33^{n}	66.02	57.85	4.4	1.93
8	1% U	35.62^{h}	8.31^{g}	40.62^{j}	9.31^{l}	44.62^{k}	12.32^{h}	1239.38^{k}	312.32^{op}	112.63^{k}	36.12^{i}	10.83^{g}	2.21^{g}	114.33^{k}	139.67^{m}	61.97	31.23	4.13	1.04
9	1% Gy	35.62^{h}	8.31^{g}	41.62^{h}	10.01^{i}	45.62^{h}	13.25^{f}	1692.37^{e}	768.32^{d}	114.33^{j}	35.31^{k}	17.89^{c}	4.12^{c}	85.39^{m}	190.63^{f}	84.62	76.83	5.64	2.56
10	2% Gy	36.62^{f}	8.61^{f}	43.63^{e}	9.62^{k}	47.62^{d}	12.63^{g}	1570.32^{h}	445.41^{m}	115.33^{i}	35.00^{l}	9.63^{h}	3.72^{d}	162.33^{d}	120.62^{op}	78.52	44.54	5.23	1.49
11	2% KH	35.62^{h}	8.12^{h}	40.61^{j}	9.33^{l}	44.47^{l}	12.32^{h}	2273.35^{d}	772.65^{c}	122.33^{d}	41.62^{b}	9.32^{i}	1.91^{h}	244.33^{b}	400.31^{d}	113.67	77.26	7.58	2.57
12	2.5% KH	35.48^{h}	8.32^{g}	40.63^{j}	9.31^{l}	44.62^{k}	12.32^{h}	2495.31^{b}	803.16^{b}	123.62^{b}	40.63^{c}	18.12^{b}	1.73^{i}	141.63^{g}	451.61^{c}	124.77	80.31	8.32	2.68
13	10 ppm G	36.59^{f}	8.65^{f}	45.62^{b}	11.32^{f}	49.62^{b}	14.25^{e}	960.33^{n}	540.66^{l}	119.33^{h}	40.12^{d}	7.65^{j}	1.02^{j}	126.33^{j}	511.65^{b}	48.02	54.06	3.2	1.8
14	20 ppm G	36.33^{g}	8.62^{f}	46.32^{a}	11.65^{e}	50.39^{a}	15.25^{d}	820.33^{op}	431.66^{n}	119.68^{g}	40.62^{c}	5.11^{l}	0.82^{k}	160.62^{e}	526.31^{a}	41.02	43.16	2.73	1.44
15	Control	36.45^{fg}	8.61^{f}	41.39^{i}	9.67^{j}	45.05^{j}	12.31^{h}	1340.35^{i}	570.35^{j}	112.33^{l}	35.32^{k}	9.43^{i}	3.12^{f}	143.15^{f}	190.31^{g}	67.02	57.03	4.47	1.90
SE m (±)		0.362	0.406	0.477	0.383	0.479	0.449	142.535	39.147	1.095	0.639	1.348	0.334	14.280	37.403				
CD (0.05)		0.226	0.092	0.162	0.030	0.137	0.218	0.117	0.040	0.071	0.133	0.122	0.133	0.106	0.038				

I: *P. cystidiosus* (non-coremial), II: *P. opuntiae*

WB: Wheat bran, RB: Rice bran, NC: Neem cake, U: Urea, Gy: Gypsum, KH: Potassium dihydrogen phosphate, G: Gibberellic acid, *Average of four replications, each replication denotes 5 beds, Means followed by similar superscripts are not significantly different at 5 % level

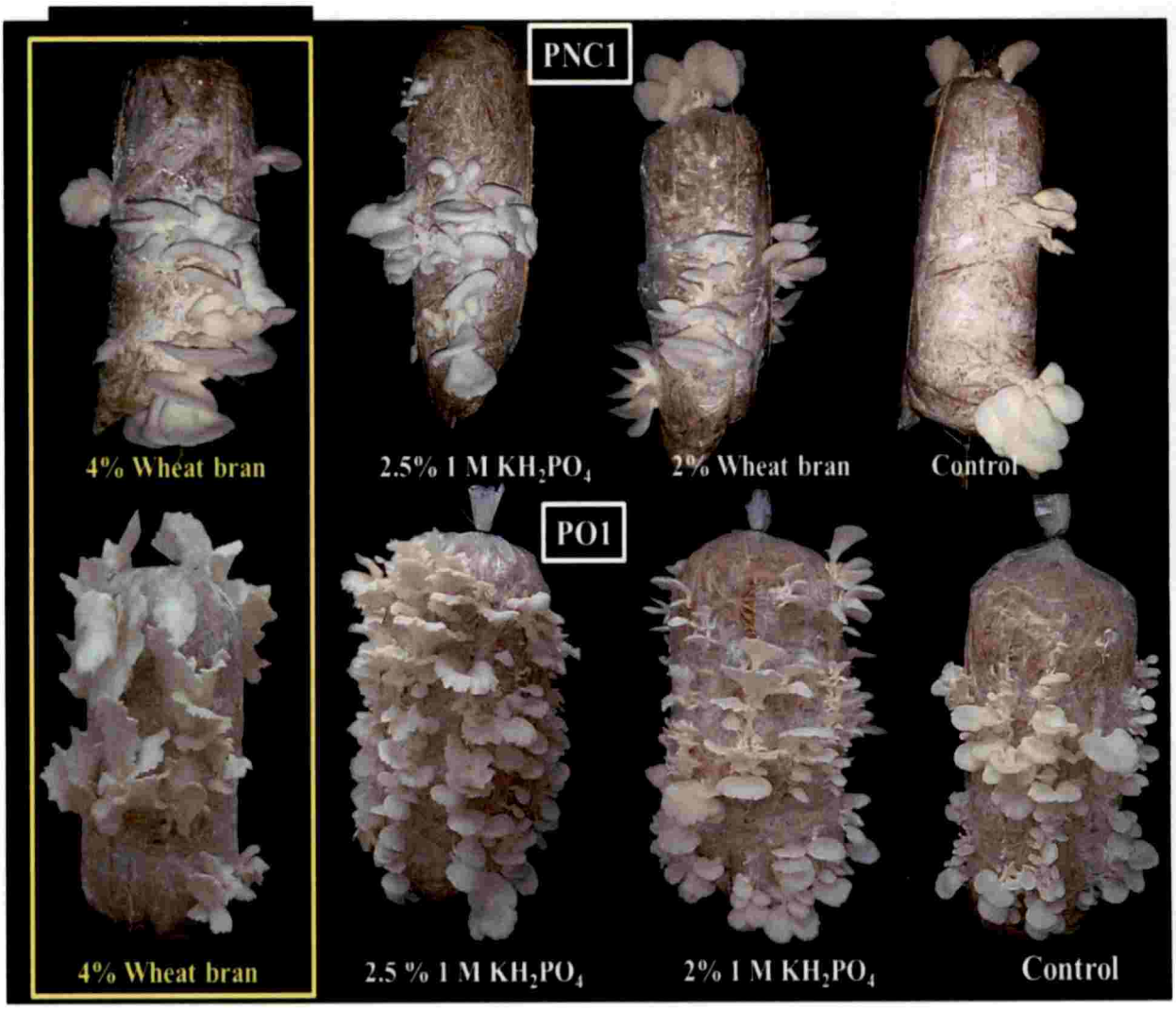


Plate 26. Influence of amended paddy straw on yield parameters of *Pleurotus* spp.

Table 22. Influence of amended rubber wood sawdust on yield parameters of *Pleurotus* spp.

Sl. No.	Trts.	Days for complete spawn run*		Days for pinhead formation*		Days for first harvest*		Total yield per bed from three harvests (g)*		Total crop period (days)*		Average weight of sporocarp (g)*		Number of sporocarps*		Biological efficiency (%)*	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
1	2% WB	48.23^{b}	14.35^{bc}	54.30^{d}	16.38^{b}	58.33^{d}	19.05^{d}	3802.32^{c}	835.30^{d}	145.31^{b}	50.30^{f}	21.85^{g}	4.75^{f}	173.33^{f}	173.38^{g}	190.1	83.5
2	4% WB	48.62^{a}	14.25^{c}	54.62^{c}	16.32^{b}	58.62^{c}	19.50^{c}	3815.41^{b}	913.65^{a}	145.31 b	51.45^{d}	36.28^{a}	4.85^{e}	106.30^{m}	185.32^{d}	190.7	91.3
3	2% RB	47.30^{d}	10.62^{f}	53.32^{f}	11.65^{f}	57.32^{g}	15.05^{h}	2915.11^{g}	619.35	143.30^{e}	49.40^{g}	15.12^{j}	3.80^{g}	193.29^{e}	162.20^{j}	145.7	61.9
4	4% RB	47.62^{c}	10.68^{f}	53.62^{e}	11.73^{f}	57.62^{f}	14.65i	2960.08^{f}	730.05^{g}	143.65^{d}	50.60^{e}	22.42^{f}	6.70^{a}	132.31^{j}	110.10^{n}	148.0	73.0
5	2% NC	46.30^{f}	13.23^{e}	52.35^{j}	14.35^{e}	56.62^{h}	18.38^{f}	2620.10^{j}	861.35^{c}	142.62^{f}	52.10^{c}	25.55^{d}	5.75^{b}	102.31^{n}	153.25^{k}	131.0	86.1
6	4% NC	46.68^{e}	13.58^{d}	53.00^{h}	14.65^{d}	57.63^{f}	18.65^{e}	2692.05^{h}	913.80^{a}	142.31^{g}	53.65^{b}	21.35^{h}	6.72^{a}	127.33^{k}	136.30^{l}	134.6	91.3
7	0.5% U	45.22^{ij}	14.40^{b}	51.63^{k}	16.35^{b}	56.30^{j}	18.35^{f}	2371.15^{l}	600.30^{j}	136.63^{j}	43.60^{k}	14.49^{k}	5.28^{d}	163.37^{g}	113.05^{m}	118.5	60.0
8	1% U	45.62^{h}	14.65^{a}	51.62^{k}	16.70^{a}	56.49^{i}	18.60^{e}	2171.32^{m}	590.50^{k}	136.12^{k}	43.30^{l}	10.25^{l}	3.55^{h}	213.35^{d}	168.25^{i}	108.5	59.0
9	1% Gy	46.10^{g}	14.35^{bc}	52.62^{i}	16.33^{b}	57.63^{f}	18.32^{f}	2680.45^{i}	780.70^{f}	138.62^{i}	45.35^{h}	19.12^{i}	5.65^{c}	140.32^{h}	136.25^{l}	134.0	78.0
10	2% Gy	45.62^{h}	14.28^{c}	53.15^{g}	16.38^{b}	58.00^{e}	18.30^{f}	2442.30^{k}	472.45^{m}	139.62^{h}	44.10^{j}	9.25^{m}	2.60^{j}	271.32^{b}	180.20^{f}	122.1	47.2
11	2% KH	45.15^{j}	10.30^{g}	51.20^{l}	11.30^{g}	56.00^{k}	14.28^{k}	3172.08^{d}	821.50^{e}	144.33^{c}	53.60^{b}	23.62^{e}	3.20^{i}	134.32^{i}	183.05^{e}	158.6	82.1
12	2.5% KH	45.25^{ij}	10.30^{g}	51.30^{l}	11.38^{g}	56.00^{k}	14.45^{j}	3855.12^{a}	913.30^{b}	145.63^{a}	56.38^{a}	35.88^{b}	3.80^{g}	107.31^{l}	239.35^{c}	192.7	91.3
13	10ppm G	45.57^{h}	14.55^{a}	56.66^{b}	16.65^{a}	61.67^{b}	20.10^{b}	1930.40^{n}	549.70^{l}	142.31^{g}	42.10^{n}	7.48^{n}	1.35^{k}	257.33^{c}	418.25^{b}	96.5	54.9
14	20ppm G	45.53^{h}	14.35^{bc}	57.15^{a}	16.35^{b}	62.00^{a}	20.30^{a}	1730.35^{op}	430.32^{n}	142.63^{f}	42.65^{m}	5.92^{op}	0.92^{l}	289.37^{a}	467.50^{a}	86.5	43.0
15	Control	45.30^{i}	14.25^{c}	51.31^{l}	16.05^{c}	56.31^{j}	18.10^{g}	3115.18^{e}	659.80^{h}	136.67^{j}	44.35^{i}	29.32^{c}	3.85^{g}	107.35^{l}	170.20^{h}	155.7	65.9
SE m (±)		0.298	0.451	0.479	0.563	0.478	0.533	170.243	42.430	0.854	1.227	2.468	0.449	16.394	26.870		
CD (0.05)		0.114	0.106	0.127	0.104	0.120	0.122	0.104	0.157	0.098	0.118	0.176	0.091	0.059	0.315		

I: *P. cystidiosus* (non-coremial), II: *P. opuntiae*, WB: Wheat bran, RB: Rice bran, NC: Neem cake, U: Urea, Gy: Gypsum, KH: Potassium dihydrogen phosphate, G: Gibberellic acid, *Average of four replications, each replication denotes 5 beds, Means followed by similar superscripts are not significantly different at 5 % level

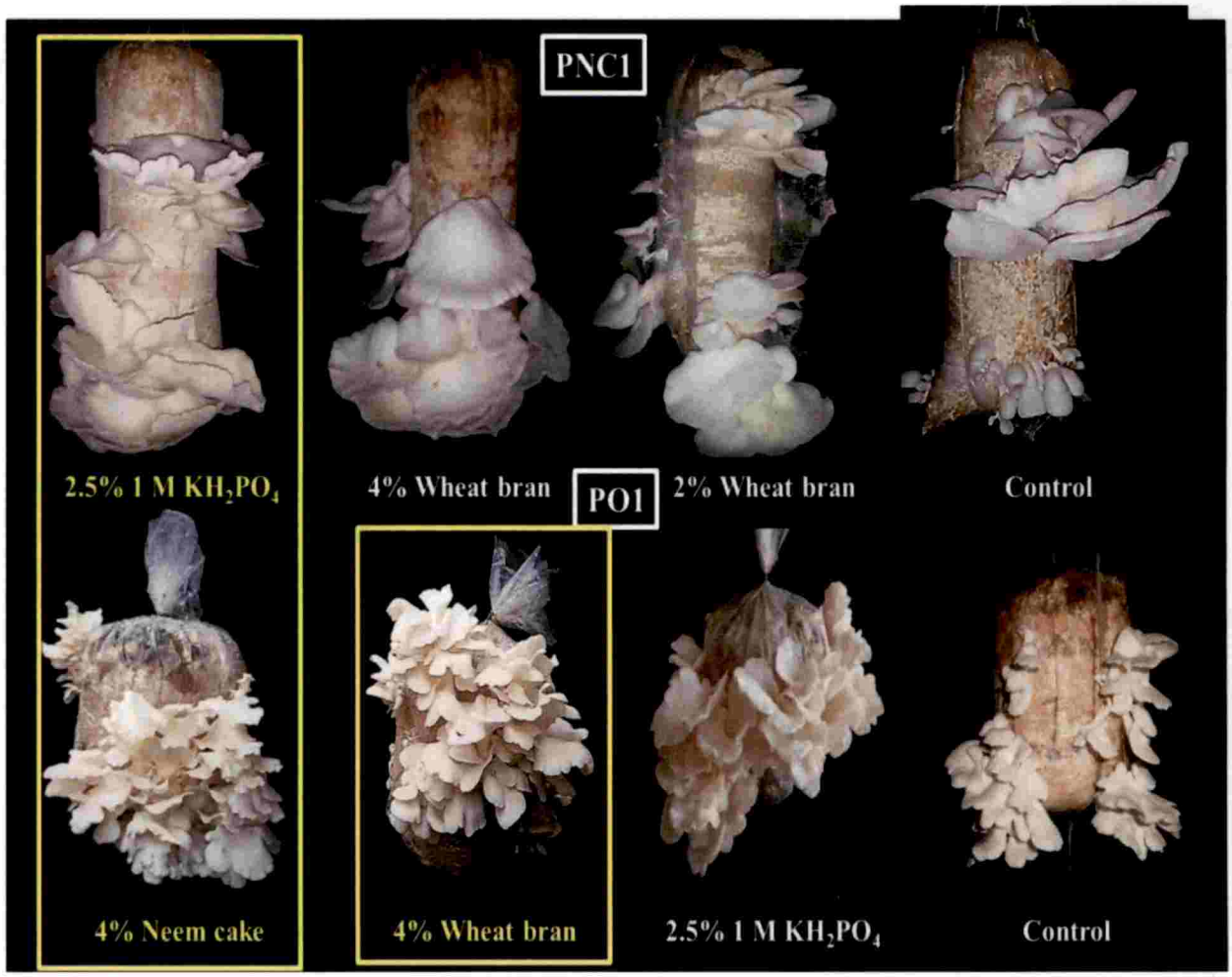


Plate 27. Influence of amended rubber wood sawdust on yield parameters of *Pleurotus* spp.

190.77 per cent, 145.31 days) and wheat bran 2 per cent (3802.32 g, 190.12 per cent, 145.31 days). Average weight of sporocarp was recorded the maximum for wheat bran 4 per cent (36.28 g) followed by 1 *M* KH_2PO4 2.5 per cent (35.88 g) and rubber wood sawdust alone (29.32 g). Maximum number of sporocarps was recorded with GA 20 ppm (289.37), followed by gypsum 2 per cent (271.32) and GA 10 ppm (257.33) (Table 22 and Plate 27).

When *P. cystidiosus* (non-coremial) was cultivated in coirpith, minimum time for spawn run was recorded with 1 *M* KH_2PO_4 2.5 per cent spray (51.12 days) and 1 *M* KH_2PO_4 2 per cent spray (51.25 days); followed by GA 20 ppm (51.32 days) and gypsum 1 per cent (51.38 days). Rice bran 2 per cent took the minimum time for pinhead formation and first harvest (56.39 and 59.25 days), followed by neem cake 2 per cent (56.67 and 59.63 days) and rice bran 4 per cent (57.31 and 60.41 days). Total yield from three harvests, BE, crop period and average weight of sporocarp were recorded maximum in beds amended with wheat bran 4 per cent (751.38 g, 37.57 per cent, 111.29 days, 13.12 g) followed by wheat bran 2 per cent (463.28 g, 23.16 per cent, 110.29 days, 11.20 g), rice bran 4 per cent (460.29 g, 23.01 per cent, 109.38 days, 9.82 g). Maximum number of sporocarps was recorded with wheat bran 4 per cent (57.32), followed by rice bran 2 per cent (48.40) and rice bran 4 per cent (47.31) (Table 23 and Plate 28).

4.10.2. Evaluation of amendments for mushroom production of *P. opuntiae*

When *P. opuntiae* was cultivated in paddy straw minimum time for spawn run, pinhead formation and first harvest was recorded with 1 *M* KH_2PO_4 2 per cent spray (8.12 days, 9.33 days and 12.32 days) followed by 1 *M* KH_2PO_4 2.5 per cent spray (8.32 days, 9.31 days and12.32 days) and urea 1 per cent (8.31 days, 9.30 days and 12.32 days). Total yield per bed from three harvests and biological efficiency was recorded maximum with wheat bran 4 per cent (806.05 g, 80.60 per cent), followed by 1 *M* KH_2PO_4 2.5 per cent spray (803.16 g, 80.31 per cent) and 1 *M* KH_2PO_4 2 per cent spray (772.65 g, 77.26 per cent). Maximum crop

period was recorded with 1 M KH_2PO_4 2.5 per cent spray (42.00 days), followed by 1 *M* KH_2PO_4 2 per cent spray (41.62 days) and GA 20 ppm spray (40.62 days). Average weight of sporocarp was found maximum for neem cake 4 per cent (4.70 g) followed by urea 0.5 per cent (4.41 g) and gypsum 1 per cent (4.12 g). Maximum number of sporocarps was noticed in beds amended with GA 20 ppm (526.31), followed by GA 10 ppm (511.65), 1 *M* KH_2PO_4 2.5 per cent (451.61) and 1 *M* KH_2PO_4 2 per cent (400.31) (Table 21 and Plate 26).

When *P. opuntiae* was cultivated in rubber wood sawdust minimum time for spawn run, pinhead formation and first harvest was taken with 1 *M* KH_2PO_4 2 per cent (10.3 days, 11.3 days and 14.28 days), followed by 1 *M* KH_2PO_4 2.5 per cent (10.3 days, 11.38 days and 14.45 days) and rice bran 4 per cent (10.68 days, 11.73 days and 14.65 days). Total yield per bed from three harvests and biological efficiency was maximum for neem cake 4 per cent (913.80 g, 91.38 per cent) and wheat bran 4 per cent (913.65 g, 91.37 per cent) followed by 1 *M* KH_2PO_4 2.5 per cent spray (913.30 g, 91.33 per cent) and neem cake 2 per cent (861.35 g, 86.13 per cent). Maximum crop period was recorded with 1 *M* KH_2PO_4 2.5 per cent (56.38 days), followed by 1 *M* KH_2PO_4 2 per cent (53.60 days) and neem cake 4 per cent (53.65 days); and neem cake 2 per cent (52.10 days). Average weight of sporocarp was found the maximum for neem cake 4 per cent (6.72 g) and rice bran 4 per cent (6.70 g) followed by neem cake 2 per cent (5.75 g) and gypsum 1 per cent (5.65 g). Maximum number of sporocarps was formed with GA 20 ppm (467.50), followed by GA 10 ppm (418.25) and 1 *M* KH_2PO_4 2.5 per cent spray (239.35) (Table 22 and Plate 27).

When *P. opuntiae* was cultivated in coirpith minimum time for spawn run, pinhead formation and first harvest was taken with 1 *M* KH_2PO4 2 per cent (10 days, 11 days and 13.32 days) followed by 1 *M* KH_2PO4 2.5 per cent (10.15 days,11.05 days and 13.25 days) and urea 1 per cent (10.37 days, 11.25 days and 13.62 days). The maximum yield per bed from three harvests and BE were recorded for wheat bran 4 per cent (460.31 g, 46.03 per cent), followed by 1 M

Table 23. Influence of amended coirpith on yield parameters of *Pleurotus* spp.

Sl. No.	Treatments	Days for complete spawn run*		Days for pinhead formation*		Days for first harvest*		Total yield per bed from three harvests (g)*		Total crop period (days)*		Average weight of sporocarp (g)*		Number of sporocarps*		Biological efficiency (%)*	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
1	2% WB	55.30^{b}	10.33^{ef}	59.31^{b}	11.31^{e}	62.30^{b}	14.35^{e}	463.28^{b}	254.72^{f}	110.29^{b}	30.32^{g}	11.20^{b}	3.55^{c}	42.25^{d}	72.67^{m}	23.16	25.47
2	4% WB	56.22^{a}	11.05^{c}	60.29^{a}	12.12^{c}	63.32^{a}	13.63^{f}	751.38^{a}	460.31^{a}	111.29^{a}	31.15^{e}	13.12^{a}	4.27^{a}	57.32^{a}	109.63^{g}	37.57	46.03
3	2% RB	52.45^{d}	10.22^{fg}	56.39^{f}	11.37^{e}	59.25^{f}	14.32e	410.38^{d}	146.40^{j}	108.30^{e}	30.62^{f}	9.51^{d}	1.93^{f}	48.40^{b}	76.63^{l}	20.52	14.64
4	4% RB	53.28^{c}	11.34^{b}	57.31^{d}	12.35^{b}	60.41^{d}	14.33^{e}	460.29^{c}	408.32^{d}	109.38^{c}	31.05^{e}	9.82^{c}	3.51^{c}	47.31^{c}	117.31^{e}	23.01	40.83
5	2% NC	52.30^{d}	11.23^{b}	56.67^{e}	12.25^{bc}	59.63^{e}	16.05^{c}	241.32^{f}	127.62^{k}	108.62^{d}	32.10^{d}	7.37^{e}	1.27^{i}	33.22^{f}	101.32^{j}	12.07	12.76
6	4% NC	53.35^{c}	11.67^{a}	57.68^{c}	13.62^{a}	60.67^{c}	17.63^{a}	253.65^{e}	437.33^{c}	108.31^{e}	33.10^{b}	7.37^{e}	4.27^{a}	34.30^{e}	104.32^{i}	12.68	43.73
7	0.5% U	52.40^{d}	10.61^{d}	-	11.37^{e}	-	14.25e	-	123.33^{l}	-	29.31^{h}	-	2.33^{e}	-	53.63^{op}	-	12.33
8	1% U	52.15^{d}	10.37^{ef}	-	11.25^{ef}	-	13.62^{f}	-	113.45^{n}	-	29.00^{i}	-	0.87^{k}	-	134.31^{d}	-	11.34
9	1% Gy	51.38^{efg}	10.39^{e}	-	11.31^{e}	-	14.20^{e}	-	273.15^{e}	-	31.12^{e}	-	3.82^{b}	-	70.65^{n}	-	27.31
10	2% Gy	51.62^{ef}	10.62^{d}	-	11.67^{d}	-	14.65^{d}	-	105.81^{op}	-	30.35^{g}	-	0.93^{j}	-	115.35^{f}	-	10.58
11	2% KH	51.25^{g}	10.00^{h}	-	11.00^{g}	-	13.32^{g}	-	161.51^{i}	-	33.05^{b}	-	2.88^{d}	-	107.31^{h}	-	16.15
12	2.5% KH	51.12^{g}	10.15^{g}	-	11.05^{fg}	-	13.25^{g}	-	459.71^{b}	-	33.37^{a}	-	1.51^{g}	-	159.37^{c}	-	45.97
13	10 ppm G	51.65^{e}	10.33^{ef}	-	12.29^{bc}	-	16.25^{bc}	-	185.40^{g}	-	32.40^{c}	-	0.93^{j}	-	187.31^{b}	-	18.54
14	20 ppm G	51.32^{fg}	10.35^{ef}	-	12.45^{b}	-	16.37^{b}	-	173.12^{h}	-	33.12^{b}	-	0.72^{l}	-	220.35^{a}	-	17.31
15	Control	52.15^{d}	10.31^{ef}	-	11.37^{e}	-	13.33^{g}	-	116.63^{m}	-	30.31^{g}	-	1.43^{h}	-	93.67^{k}	-	11.66

I: *P. cystidiosus* (non-coremial), II: *P. opuntiae*

WB: Wheat bran, RB: Rice bran, NC: Neem cake, U: Urea, Gy: Gypsum, KH: Potassium dihydrogen phosphate, G: Gibberellic acid, *Average of four replications, each replication denotes 5 beds, Means followed by similar superscripts are not significantly different at 5 % level

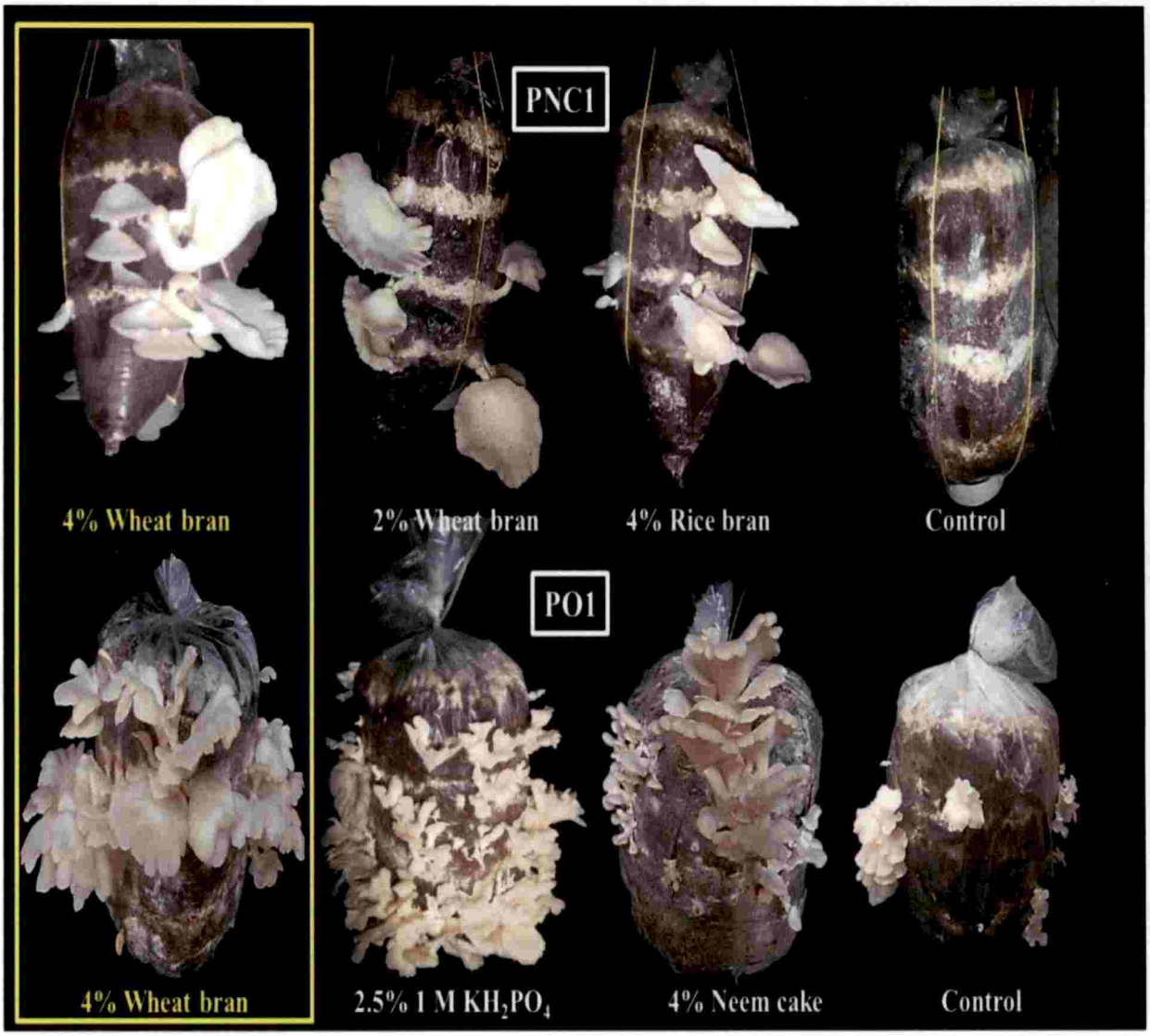


Plate 28. Influence of amended neopeat on yield parameters of *Pleurotus* spp.

Table 24. Pathogens and pests observed in mushroom beds of *Pleurotus* spp.

Sl. No.	Substrates	Pathogens	Pests
1.	Paddy straw	*Trichoderma* sp., *Aspergillus* sp., *Coprinus* sp., *Penicillium* sp. *Bacillus* sp.	Phorid flies *(Megaselia* spp.), Staphylinid beetles (*Scaphisoma* sp.), Springtails (*Seira* spp.) , Black ants
2.	Rubber wood sawdust	*Aspergillus* sp., *Trichoderma* sp., *Coprinus* spp. *Penicillium* sp.	Phorid flies (*Megaselia* spp.), Staphylinid beetles (*Scaphisoma* sp.), Springtails (*Seira* spp.), Mites
3.	Coirpith	*Trichoderma* sp., *Coprinus* spp.	Staphylinid beetles (*Scaphisoma* sp.), Black ants

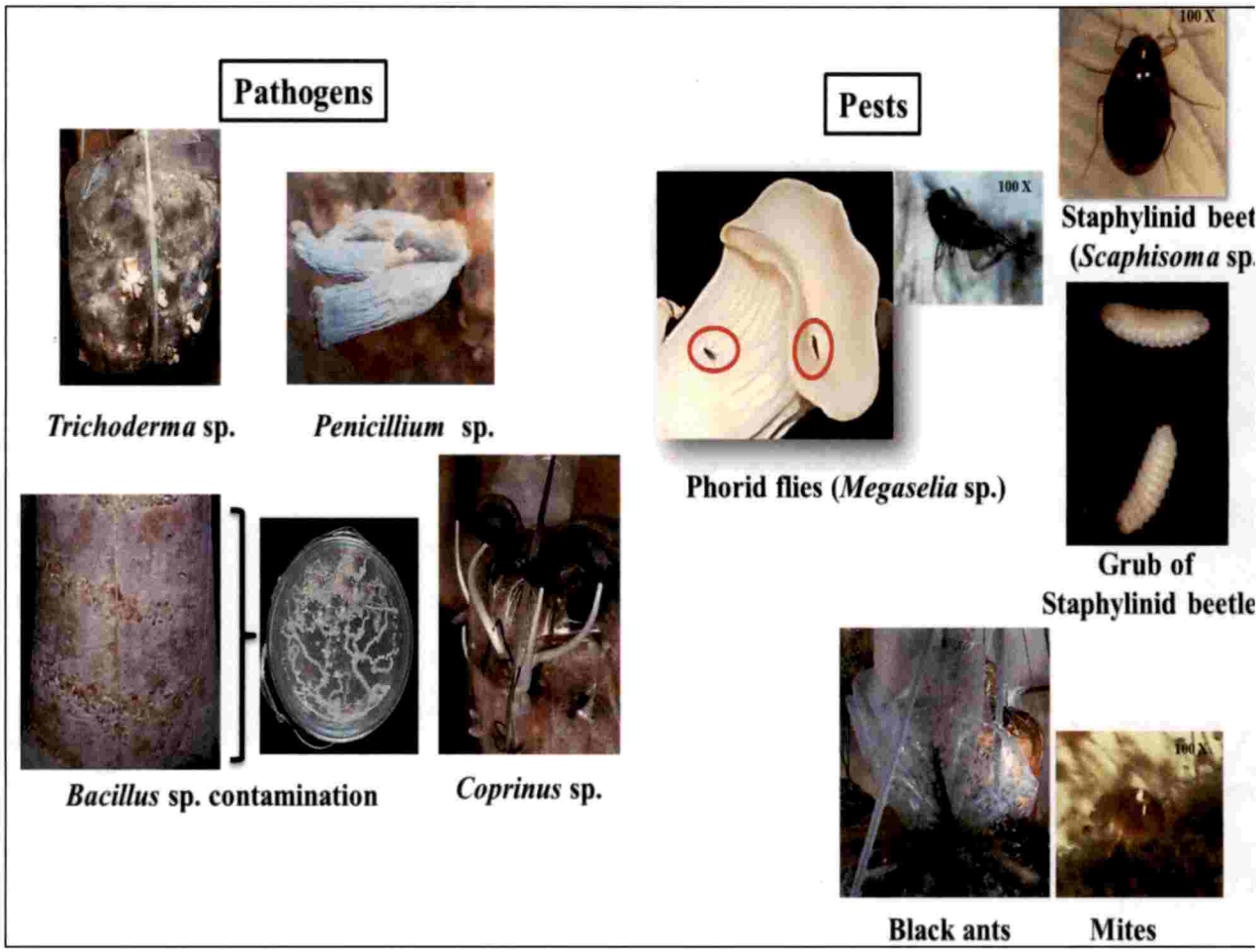


Plate 29. Pathogens and pests observed in beds of *Pleurotus* spp.

KH_2PO4 2.5 per cent (459.71 g, 45.97 per cent), and neem cake 4 per cent (437.33 g, 43.73 per cent). Maximum crop period was recorded with 1 *M* KH_2PO4 2.5 per cent spray (33.37 days) followed by 1 *M* KH_2PO_4 2 per cent spray (33.05 days), GA 20 ppm (33.12 days) and neem cake 4 per cent (33.10 days). Average weight of sporocarp was recorded the maximum for neem cake 4 per cent (4.27 g) and wheat bran 4 per cent (4.27 g), followed by gypsum 1 per cent spray (3.82 g) and; wheat bran 2 per cent (3.55 g) and rice bran 4 per cent (3.51 g). Maximum number of sporocarps was recorded in beds amended with GA 20 ppm spray (220.35), followed by GA 10 ppm spray (187.31) and 1 *M* KH_2PO_4 2.5 per cent spray (159.37) (Table 23 and Plate 28).

Major insect pests identified from different substrates of *P. cystidiosus* were *viz*., phorid flies *(Megaselia* spp.) and staphylinid beetles; in paddy straw and sawdust of rubber tree. Mites were also recorded from the sawdust of rubber tree. *Trichoderma* sp., *Aspergillus* sp., *Coprinus* sp. and *Bacillus* sp. were the major contaminants in paddy straw; and *Aspergillus* sp. and *Penicillium* sp. in sawdust of rubber tree (Plate 29). The major insect pests were identified from different substrates of *P. opuntiae viz*., phorid flies (*Megaselia* spp.), springtails (*Seira* spp.) and black ants in paddy straw, phorid flies (*Megaselia* spp.), springtails (*Seira* spp.), staphylinid beetles and mites in sawdust of rubber tree. *Trichoderma* sp., *Aspergillus* sp. and *Coprinus* sp. were the major fungal contaminants found in paddy straw and sawdust of rubber tree. When coirpith was used as substrate, staphylinid beetles and black ants were observed as the major pests, while the fungal contaminants identified were *Trichoderma* spp. and *Coprinus* spp. (Table 24).

4.11. DEVELOPMENTAL MORPHOLOGY OF PLEUROTUS SPP.

The sporocarps of *P. cystidiosus* (coremial) took an average of 5 days from the day of pinhead formation to complete maturity. The pinheads were formed 46.50 days after spawning. Pileus of the pinhead was brown in colour, soft textured, bulged, circular of size 0.67 cm x 0.66 cm and stipe was brown, reduced,

of length 0.05 cm, with an average weight of 1.20 g. Three days after pinhead formation, pileus turned brown in colour with irregular margin, of size 12.80 cm x 5.80 cm and straight, thick, white stipe of length 3.10 cm, with an average weight of 33.68 g. On the fifth day, the mushroom attained full maturity; and light brown, large, fleshy sporocarps with irregular margin were formed. As the pileus opened fully, the length of the stipe was reduced and straight, white, thick, reduced stipe of length 2.5 cm was formed. Thus, mature sporocarps were formed 50.75 days after spawning, with pileus of size 17.01 cm x 7.68 cm and average weight of 46.72 g (Table 25 and Plate 30).

The sporocarps of *P. cystidiosus* (non-coremial) took an average of 5 days from the day of pinhead formation to complete maturity. Pinheads were formed 41.25 days after spawning. The pileus of the pinhead was soft textured, white in colour, circular with clear margin of size 0.68 cm x 0.55 cm and white stipe of length 0.88 cm, with weight of 1.07 g. After 3 days of pin head formation, the white pileus attained regular margin and got bulged at the centre with size of 6.60 cm x 6.01 cm and curved white stipe of length 3.9 cm. After 45 days of spawning, the mushroom attained full maturity and fleshy, pure white, large sporocarps with regular margin and flattened pileus were formed. The mature sporocarps had pileus of size 14.10 cm x 11.08 cm and stipe of length 3.10 cm with average weight of 31.09 g. At 46.25 days of spawning, mushroom turned overmature and dull white, large sporocarps with regular margin and flattened pileus of size 14.20 cm x11.20 cm was formed with short curved stipe of length 2.89 cm (Table 25 and Plate 31).

The sporocarps of *P. opuntiae* took an average of 5 days from the day of pinhead formation to complete maturity. Pinhead was formed 8.75 days after spawning. The pileus of the pinhead was white in colour, circular, soft textured, with clear margin of size 0.67 cm x 0.52 cm. The stipe was of length 0.91 cm with an average weight of 0.09 g. Two days after pinhead formation the white pileus attained regular margin with size of 3.20 cm x 2.30 cm and average weight of 0.67 g. On the fifth day *i.e.* 12 days after spawning the fleshy, white, small oyster

Table 25. Developmental morphology of *Pleurotus* spp.

Sl. No	Stages	Dimension of pileus (l cm x b cm)*			Length of stipe (cm)*			Weight of sporophore (g)*			Days after spawning*			Morphological characters		
		I	II	III	I	II	III	I	II	III	I	I	III	I	II	III
1	I	0.67 x 0.66	0.68 x 0.55	0.67 x 0.52	0.05	0.88	0.91	1.20	1.07	0.09	46.50	41.25	8.75	Brown, circular, bulged pileus. Brown, reduced stipe.	White pileus and stipe, pileus circular with clear margin.	White pileus and stipe, pileus circular with clear margin.
2	II	3.91 x 3.20	0.70 x 0.59	1.70 x 1.20	1.33	0.91	1.20	5.86	2.10	0.23	47.25	42.25	9.00	Brown pileus, with irregular margin. Straight, white stipe.	White, circular pileus, with clear margin. Curved, white stipe.	White, circular pileus, with clear margin. Straight, white stipe.
3	III	5.90 x 5.00	3.10 x 2.80	3.20 x 2.30	1.53	1.90	1.40	15.49	6.57	0.67	48.00	43.00	9.75	Brown, pileus with irregular margin. Straight, thick, white stipe.	White pileus with regular margin. Curved, white stipe.	White coloured pileus with regular margin.
4	IV	12.80 x 5.80	6.60 x 6.01	4.20 x 3.60	3.10	3.90	1.73	33.68	14.61	2.81	49.00	44.00	11.00	Brown pileus with irregular margin and and flat surface. Straight, thick, white stipe.	White pileus with regular margin and bulged at the centre. Curved, white stipe.	White pileus with irregular margin and depression in centre.
5	V	17.01 x 7.68	14.10 x 11.08	6.85 x 5.20	2.50	3.10	2.10	46.72	31.09	6.84	50.75	45.00	12.00	Light brown, large, fleshy, sporocarps with irregular margin and flat surface. Straight, white, thick, reduced stipe.	Fleshy, pure white, large sporocarps, with regular margin and flattened pileus. Reduced, curved stipe	Fleshy,pure white, small sporocarps, with irregular margin and depression in the centre.

* Average of 20 replications

I: *P. cystidiosus* (coremial), II: *P. cystidiosus* (non-coremail), III: *P. opuntiae*

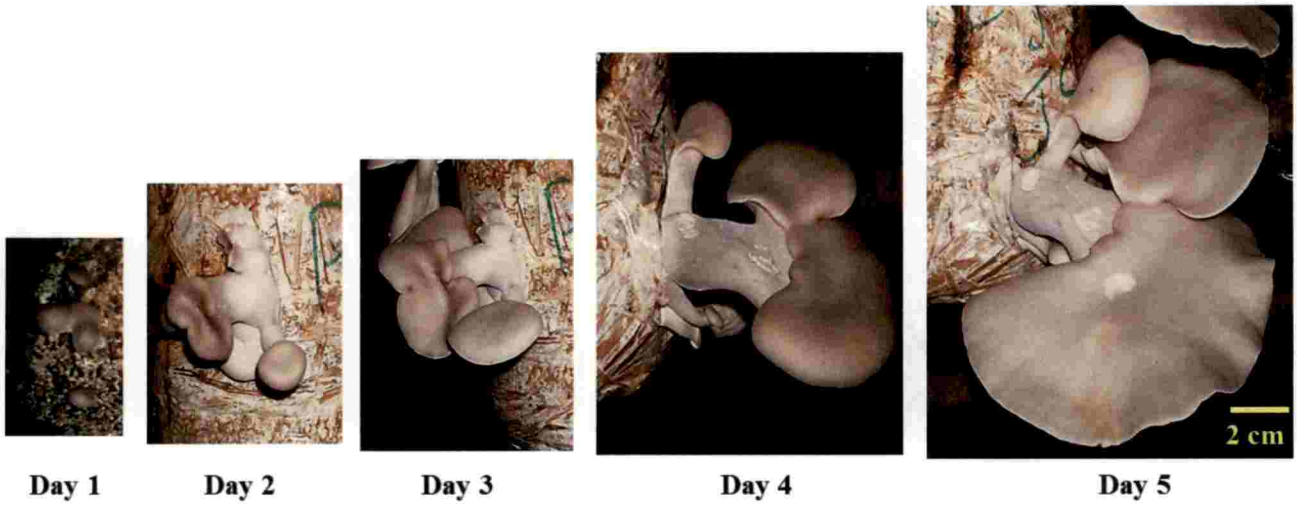


Plate 30. Developmental morphology of *P. cystidiosus* (coremial)

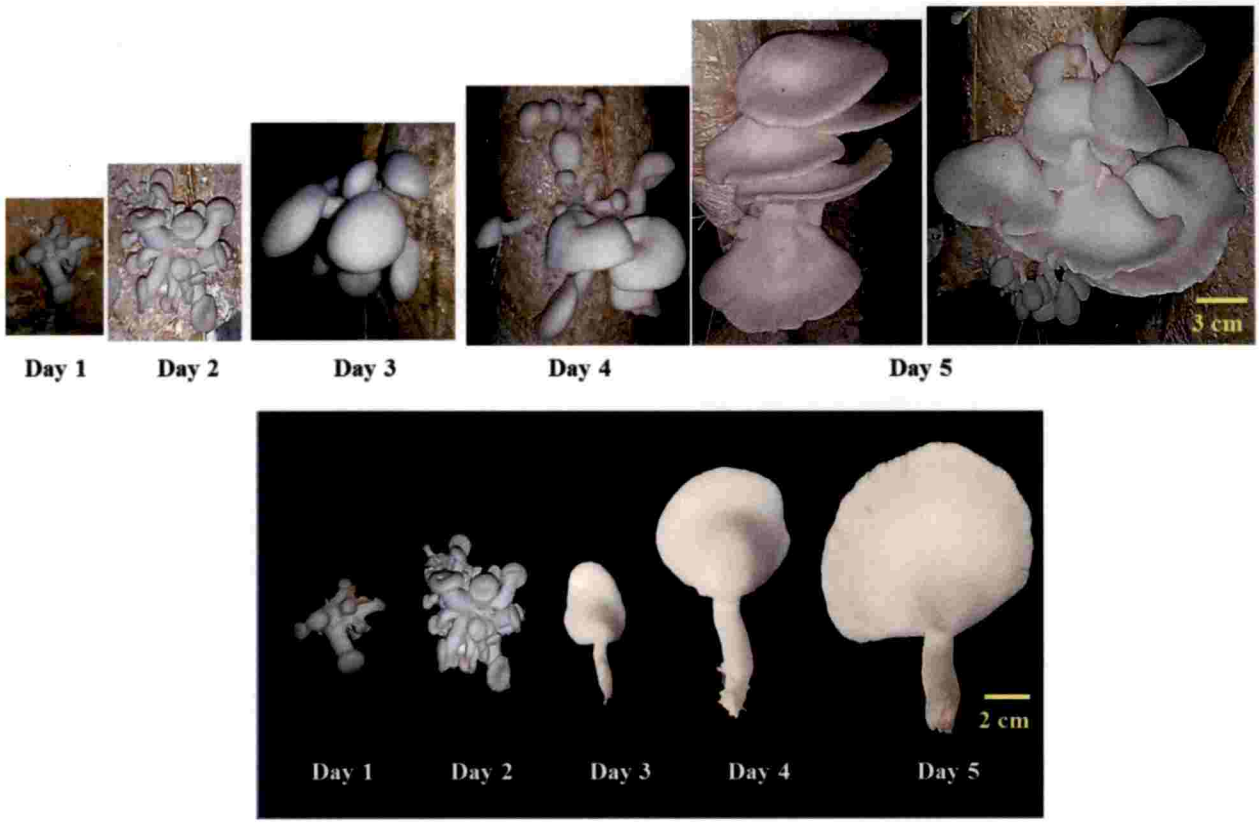


Plate 31. Developmental morphology of *P. cystidiosus* (non-coremial)

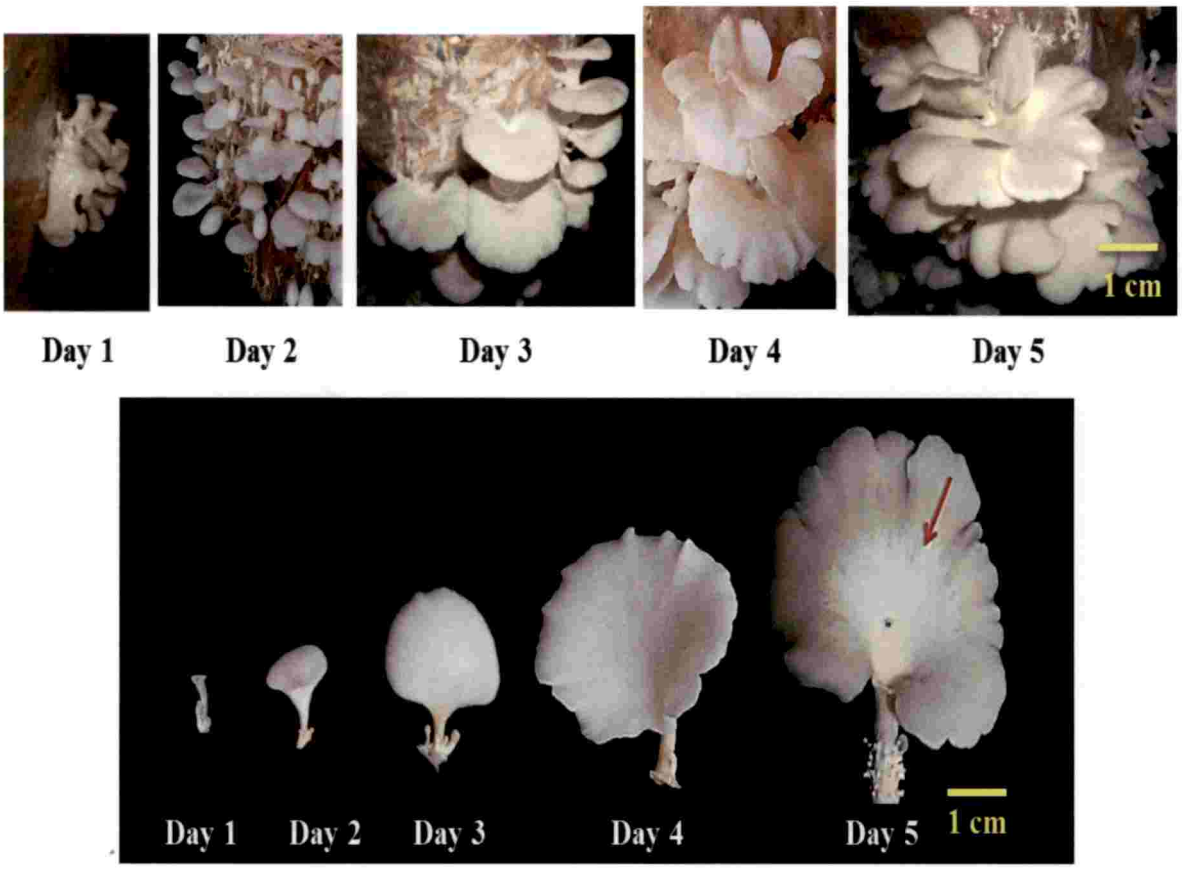


Plate 32. Developmental morphology of *P. opuntiae*

shaped sporocaps with irregular/fringed margin and depression in the centre of size 6.85 x 5.20 cm and stipe length of 2.10 cm, with weight of 6.84 g was formed. After 13.13 days of spawning, pileus opened fully, and dull white, sporocarps with irregular margin; and thick white radiating patch on the centre of the pileus was formed. The over mature sporocarps were coarse in texture of size 7.35 cm x 5.44 cm, with stipe length of 2.22 cm and average weight of 8.93 g (Table 25 and Plate 32).

Sporocarps of the *Pleurotus* spp. were normally produced in clusters and rarely seen as single sporocarp. Even within a bunch the sporocarps differed in their size and morphology. The maturity of sporocarps of *Pleurotus* spp. were greatly influenced by factors such as temperature, humidity and aeration. High temperature and low humidity inside the cultivation room lead to the drying of pinheads on the very next day of formation.

4.12. COMPARATIVE PERFORMANCE

P. florida and *P. eous* are the widely cultivated mushroom species in Kerala state. Hence, they were selected for comparative studies with *P. cystidiosus* (coremial and non-coremial) and *P. opuntiae* respectively.

4.12.1. Spawn production

Sorghum grains (amended with yeast 1 per cent) and paddy grains (amended with yeast 1 per cent) were used as substrates for the comparative study, as these were proved to be the best substrates from spawn studies. Comparative performance of *P. cystidiosus* (coremial and non-coremial) with *P. florida* for spawn production indicated that, minimum time for spawn run was recorded with *P. cystidiosus* (non-coremial) in paddy grains (amended with yeast 1 per cent) (5.61 days), followed by *P. florida* in sorghum grains (amended with yeast 1 per cent) (10.79 days) and *P. florida* in paddy grains (amended with yeast

Table 26. Comparative performance of *P. cystidiosus* (coremial and non-coremial) and *P. florida* for spawn production in best substrates *viz.*, sorghum (amended with 1% yeast) and paddy grains (amended with 1% yeast)

Sl. No.	Spawns	Days for spawn run*	Nature of mycelial growth	Presence of contaminants
Sorghum + 1% yeast				
1	*P. cystidiosus* coremial	17.35[b]	++++	-
2	*P. florida*	10.79[d]	++++	-
Paddy grains + 1% yeast				
1	*P. cystidiosus* coremial	23.15[a]	++++	-
2	*P. cystidiosus* non-coremial	5.61[e]	++++	-
3	*P. florida*	12.70[c]	++++	-
SE m (±)		2.977		
CD (0.05)		0.154		

* Average of four replications, Treatments are significantly different at 5 % level, ++++ : Thicker and fluffy growth +++ : Thick growth ++ : Poor growth

Table 27. Comparative performance of *P. opuntiae* and *P. eous* for spawn production in best substrates *viz.*, sorghum (amended with 1% yeast) and paddy grains (amended with yeast 1%)

Sl. No.	Spawns	*Days for spawn run	Nature of mycelial growth	Presence of contaminants
Sorghum + 1% yeast				
1	*P. opuntiae*	9.28[d]	++++	-
2	*P. eous*	10.25[c]	++++	-
Paddy grains + 1 % yeast				
1	*P. opuntiae*	11.40[b]	++++	-
2	*P. eous*	12.65[a]	++++	-
SE m (±)		0.728		
CD (0.05)		0.158		

*Average of four replications, Treatments are significantly different at 5 % level, ++++ : Thicker and fluffy growth +++ : Thick growth ++ : Poor growth

Plate 33. Spawns of *P. cystidiosus* (coremial) and *P. florida* in sorghum (amended with 1 % yeast)

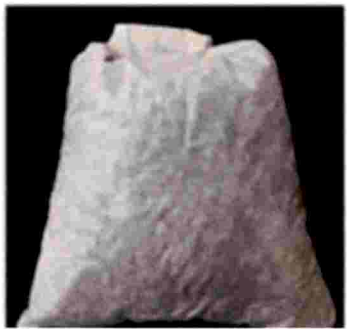


Plate 34. Spawn of *P. cystidiosus* (non-coremial) in paddy grain amended with 1% yeast

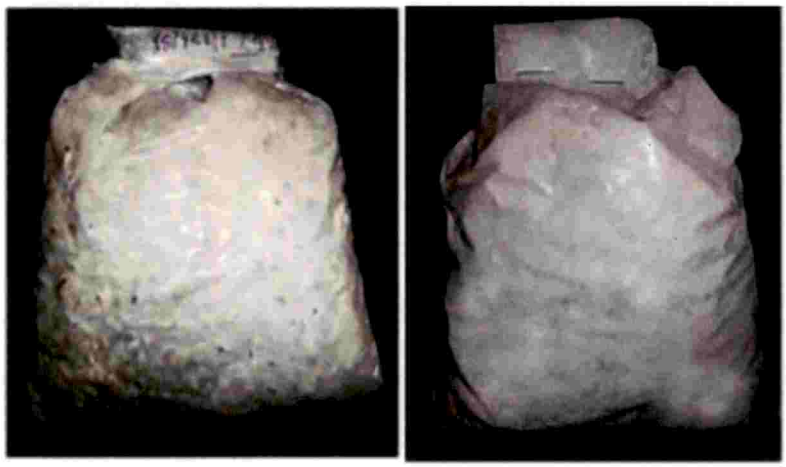


Plate 35. Spawns of *P. opuntiae* and *P. eous* in sorghum (amended with yeast 1%)

1 per cent), (12.70 days), with thicker and fluffy growth (Table 27 and Plate 33&34).Comparative performance of *P. opuntiae* and *P. eous* for spawn production, indicated that minimum time for spawn run was recorded with *P. opuntiae* in sorghum grains (amended with yeast 1 per cent) (9.28 days), followed by *P. eous* in sorghum grains (amended with yeast 1 per cent) (10.25 days) and *P. opuntiae* in paddy grains (amended with yeast 1 per cent) (11.40 days), with thicker and fluffy growth (Table 26 and Plate 35).

4.12.2. Mushroom Production

Comparative performance of *P. cystidiosus* (non-coremial) and *P. florida* for mushroom production, indicated that, time taken for spawn run (45.50 days), pin head formation (51.70 days), first flush (56.40 days), total crop period (145.30 days), average weight (35.80 g), number of sporocarps (106.90), total yield (3858.60 g) and biological efficiency (192.20 per cent) were significantly higher for *P. cystidiosus* (non-coremial) on rubber wood sawdust sprayed with 2.5% of 1*M* KH_2PO_4 than *P. florida* on the same amended medium (Table 27 and Plate 34). Comparative performance of *P. opuntiae* and *P. eous* for mushroom production indicated that time taken for spawn run (13.40 days), pin head formation (14.20 days), first flush (18.20 days), total crop period (53.50 days), maximum number of sporocarps (137.10), total yield (913.10 g) and biological efficiency (91.40 per cent) was significantly greater for *P. opuntiae*, on rubber wood sawdust amended with wheat bran 4 per cent. However average weight of sporophore was significantly greater for *P. eous* (14.20 g) on rubber wood sawdust amended with neem cake 4 per cent (Table 28&29). Common pests observed during the study were phorid flies and staphylinid beetles. The competitor moulds *viz., Trichoderma* sp., *Aspergillus* sp., *Coprinus* sp. and *Bacillus* sp. were also observed on the beds.

4.12.3. Seasonal variation in production of *P. cystidiosus* (non-coremial) and *P. opuntiae*

Table 28. Comparative performance of *P. cystidiosus* (non-coremial) vs. *P. florida* on rubber wood sawdust sprayed with $1M\ KH_2PO_4$ 2.5 %.

Sl. No.	Treatments	*TSR	*TPF	*TFF	*TCP	*W	*N	*TY	*BE
1	*P. cystidiosus* non-coremial	45.50	51.70	56.40	145.30	35.80	106.90	3858.60	192.20
2	*P. florida*	26.10	32.30	35.60	71.70	29.20	37.80	2322.10	116.40
t (0.05)		2.101**	2.101**	2.101**	2.101**	2.101**	2.101**	2.262**	

TSR: Time taken for complete spawn run (days), TPF: Time taken for pinhead formation (days), TFF: Time taken for first flush (days), TCP: Total crop period (days), W: Weight of sporocarps (g), N: Number of sporocarps, TY: Total yield from three harvest (kg), BE: Biological Efficiency (%), *Average of ten replications

Table 29. Comparative performance of *P. opuntiae* vs. *P. eous* on rubber wood sawdust separately amended with wheat bran 4 % and neem cake 4 %

Treatments	TSR*	TPF*	TFF*	TCP*	W*	N*	TY*	BE*
P. opuntiae WB	13.60^{a}	14.40^{a}	18.24^{ab}	53.40^{a}	6.20^{c}	137.60^{a}	913.60^{a}	91.36
P. eous WB	12.40^{c}	13.40^{b}	16.60^{c}	50.60^{b}	13.26^{b}	58.20^{c}	742.80^{c}	74.28
P. opuntiae NC	13.80^{a}	14.60^{a}	18.60^{a}	54.00^{a}	6.60^{c}	136.40^{a}	913.40^{a}	91.34
P. eous NC	13.00^{b}	14.20^{a}	18.00^{b}	50.80^{b}	14.20^{a}	63.2^{b}	781.60^{b}	78.16
SE m (±)	0.316	0.263	0.438	0.875	2.126	22.051	44.389	
CD (0.05)	0.596	0.701	0.591	0.763	0.637	1.572	1.279	

TSR: Time taken for complete spawn run (days), TPF: Time taken for pinhead formation (days), TFF: Time taken for first flush (days), TCP: Total crop period (days), W : Weight of sporocarps (g), N: Number of sporocarps, TY: Total yield from three harvest (kg), BE: Biological Efficiency (%), *Average of five replications.

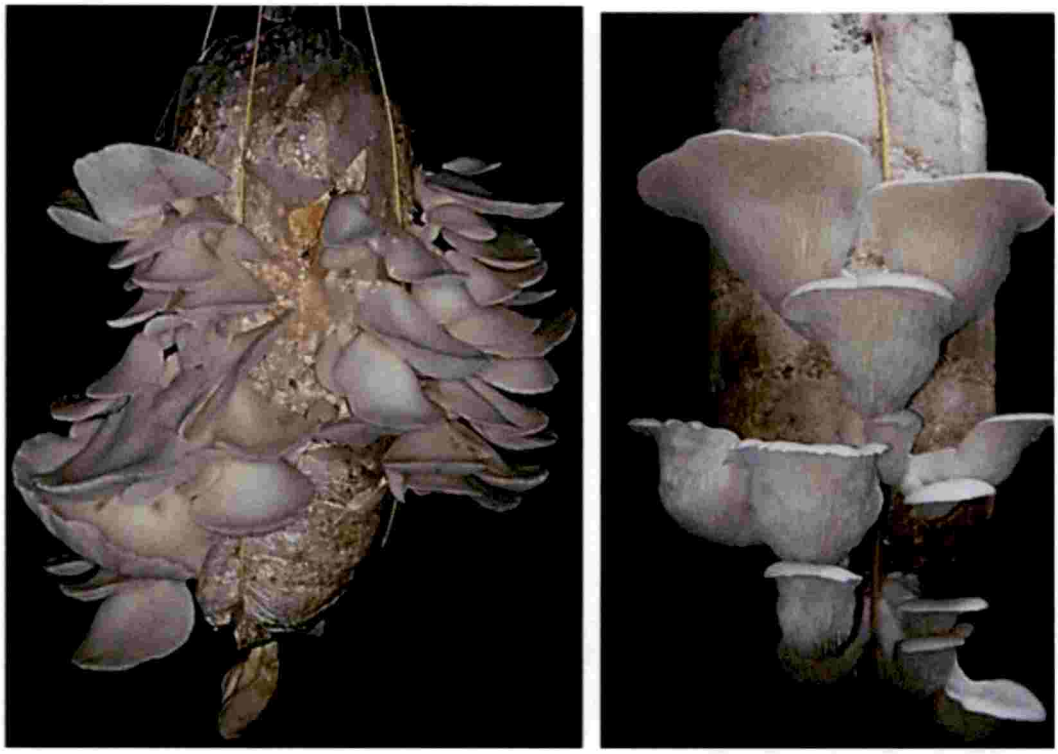


Plate 36. *P. cystidiosus* (non-coremial) and *P. florida* in rubber wood sawdust sprayed with 1*M* KH2PO4 2.5%.

Plate 37. *P. opuntiae* and *P. eous* in rubber wood sawdust amended with wheat bran 4 %

Plate 38. *P. opuntiae* and *P. eous* in rubber wood sawdust amended with neem cake 4%.

Table 30. Seasonal variation in production of *P. opuntiae* and *P. cystidiosus* (non-coremial) in Vellayani and Idukki

Variety	Seasons (2015-2017)					
	February-May		June-September		October-January	
	*First harvest (days)	*Total yield (g kg^{-1})	*First harvest (days)	*Total yield (g kg^{-1})	*First harvest (days)	*Total yield (g kg^{-1})
P. opuntiae						
Vellayani	19.30[d]	811.82[b]	18.30[d]	913.40[b]	23.25[d]	689.18[b]
Idukki	26.32[c]	712.28[c]	25.38[c]	670.25[c]	31.28[c]	610.18[c]
P. cystidiosus (non-coremial)						
Vellayani	84.38[a]	187.28[d]	71.30[a]	361.35[d]	68.20[a]	355.32[d]
Idukki	69.25[b]	1457.55[a]	56.30[b]	1927.20[a]	54.18[b]	1875.28[a]
SE m (±)	15.957	260.671	12.577	339.083	10.334	338.509
CD(0.05)	0.439	0.708	0.396	0.426	0.310	0.377

*Average of ten replications
Treatments are significantly different at 5 % level

Seasonal variation in production of *P. cystidiosus* (non-coremial) and *P. opuntiae* in Vellayani and Idukki, was carried out in three seasons *viz*., February-May, June-September and October-January and the results are presented in Table 30. In Vellayani and Idukki, *P. cystidiosus* gave the maximum yield during June-September (361.35 g and 1927.20 g), followed by October-January (355.32 and 1875.28 g) and February-May (187.28 g and 1457.55 g). In Vellayani, *P. opuntiae* gave the maximum yield during June-September (913.40 g) followed by February-May (811.82 g) and October-January (689.18 g). In Idukki, *P. opuntiae* gave the maximum yield during February-May (712.28 g), followed by June-September (670.25 g) and October-January (610.18 g). In Vellayani and Idukki, *P. opuntiae,* took the minimum time for harvest during June-September (18.3 days and 25.38 days), February-May (19.30 days and 26.32 days) and October-January (23.25 and 31.28 days). In Vellayani and Idukki, *P. cystidiosus* (non-coremial) took the mimimum time for harvest during October-January (68.20 days and 54.18 days) followed by June-September (71.30 days and 56.30 days) and February-May (84.38 days and 69.25 days). During February-May, June-September and October-January, *P. cystidiosus* (non-coremial) in Idukki gave the maximum yield (1457.55 g, 1927.20 g and 1875.28 g) followed by *P. opuntiae* in Vellayani (811.82 g, 913.40 g and 689.18 g) and *P. opuntiae* in Idukki (712.28 g, 670.25 g and 610.18 g). During February-May, June-September and October-January minimum time for first harvest was recorded with *P. opuntiae* in Vellayani (19.30 days, 18.3 days and 23.25 days) followed by *P. opuntiae* in Idukki (26.32 days, 25.38 days and 31.28 days) and *P. cystidiosus* (non-coremial) in Idukki (69.25 days, 56.30 days and 54.18 days).

4.13. PROXIMATE CONSTITUENTS OF *PLEUROTUS* SPP.

Proximate constituents of the four *Pleurotus* spp *viz., P. cystidiosus* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous* were evaluated by analysing the moisture content, carbohydrate, polysaccharides, protein, fat, fibre, ash, total

antioxidants, betacarotene, polyphenols followed by estimation of their energy value. and the results are presented in Table 31.

4.13.1. Moisture content

Highest moisture content was recorded for *P. cystidiosus* (non-coremial) (94.05 per cent), followed by *P. florida* (93.92 per cent), *P. opuntiae* (90.7 per cent) and *P. eous* (88.39 per cent).

4.13.2. Carbohydrates

P. florida showed the maximum carbohydrate content (64.94 mg g^{-1}), followed by *P. cystidiosus* (non-coremial) (58.55 mg g^{-1}) and *P. opuntiae* (46.13 mg g^{-1}) and *P. eous* (36.44 mg g^{-1}).

4.13.2.1. Polysaccharide

P. cystidiosus (non-coremial) recorded the maximum starch content (200.55 mg g^{-1}), followed by *P. florida* (90.10 mg g^{-1}), *P. eous* (52.20 mg g^{-1}) and *P. opuntiae* (7.55 mg g^{-1}). *P. florida* recorded the maximum cellulose content (60.4 mg g^{-1}), followed by *P. cystidiosus* (non-coremial) (56.11 mg g^{-1}), *P. eous* (13.32 mg g^{-1}) and *P. opuntiae* (11.27 mg g^{-1}). *P. florida* recorded the maximum reducing sugar (7.08 mg g^{-1}), followed by *P. opuntiae* (6.31 mg g^{-1}), *P. eous* (5.10 mg g^{-1}) and *P. cystidiosus* (non-coremial) (3.02 mg g^{-1}).

4.13.3. Protein content

P. cystidiosus (non-coremial) recorded the maximum protein content (30.20 mg g^{-1}) followed by *P. eous* (21.98 mg g^{-1}), *P. florida* (20.10 mg g^{-1}) and *P. opuntiae* (19.97 mg g^{-1}).

4.13.4. Fat content

Table 31. Analysis of proximate constituents of four *Pleurotus* spp.

Sl. No.	*Constituents	Moisture (%)	Carbohydrate (mg g^{-1})	Reducing sugars (mg g^{-1})	Polysaccharides		Protein (mg g^{-1})	Fat (%)	Fibre (%)	Ash (%)	Antioxidants (µg Eq.g^{-1} of ascorbic acid)	βcarotene (µg $100mg^{-1}$)	Polyphenols (mg g^{-1})	Energy (Kcal)
					Starch (mg g^{-1})	Cellulose (mg g^{-1})								
1	*P. opuntiae*	90.70^{c}	46.13^{c}	6.31^{b}	7.55^{d}	11.27^{d}	19.97^{d}	2.25^{b}	9.68^{c}	6.10^{c}	370.55^{b}	12.91^{c}	7.14^{b}	264.45^{c}
2	*P. cystidiosus* non-coremial	94.05^{a}	58.55^{b}	3.02^{d}	200.55^{a}	56.11^{b}	30.20^{a}	4.25^{a}	8.72^{d}	6.00^{d}	485.45^{a}	25.69^{a}	7.55^{a}	359.45^{a}
3	*P. florida*	93.92^{b}	64.94^{a}	7.08^{a}	90.10^{b}	60.40^{a}	20.10^{c}	1.55^{d}	10.49^{a}	10.65^{a}	112.25^{c}	8.55^{d}	1.91^{c}	337.89^{b}
4	*P. eous*	88.39^{d}	36.44^{d}	5.10^{c}	52.20^{c}	13.32^{c}	21.98^{b}	2.12^{c}	10.11^{b}	9.10^{b}	69.82^{d}	19.82^{b}	1.45^{d}	228.18^{d}

* Average of four replications, Means followed by similar superscripts are not significantly different at 5 % level

P. cystidiosus (non-coremial) recorded the maximum fat content (4.25 per cent) followed by *P. opuntiae* (2.25 per cent), *P. eous* (2.12 per cent) and *P. florida* (1.55 per cent).

4.13.5. Fibre

P. florida showed the highest fibre content (10.49 per cent) followed by *P. eous* (10.11 per cent) and *P. opuntiae* (9.68 per cent) and *P. cystidiosus* (non-coremial) (8.72 per cent).

4.13.6. Ash

P. florida showed the highest ash content (10.65 per cent) followed by *P. eous* (9.10 per cent), *P. opuntiae* (6.10 per cent) and *P. cystidiosus* (non-coremial) (6.00 per cent).

4.13.7. Total antioxidants

P. cystidiosus (non-coremial) recorded the maximum antioxidant value (485.45 µg Eq.g $^{-1}$of ascorbic acid), followed by *P. opuntiae* (370.55 µg Eq.g^{-1} of ascorbic acid), *P. florida* (112.25 µg Eq.g^{-1}of ascorbic acid) and *P. eous* (69.82 µg Eq.g^{-1}of ascorbic acid).

4.13.8. Beta carotene

P. cystidiosus (non-coremial) showed the maximum beta carotene content (25.69 µg 100mg^{-1}) followed by *P. eous* (19.82 µg 100mg^{-1}), *P. opuntiae* (12.91 µg 100mg^{-1}) and *P. florida* (8.55 µg 100mg^{-1}).

4.13.9. Polyphenol content

P. cystidiosus (non-coremial) had the maximum polyphenol content (7.55 mg g^{-1}) followed by *P. opuntiae* (7.14 mg g^{-1}), *P. florida* (1.91 mg g^{-1}) and *P. eous* (1.45 mg g^{-1}).

4.13.10. Energy

P. cystidiosus (non-coremial) had the maximum energy value (359.45 Kcal) followed by *P. florida* (337.89 Kcal), *P. opuntiae* (264.45 Kcal) and *P. eous* (228.18 Kcal).

4.14. ANTICANCEROUS ACTIVITIES OF *PLEUROTUS* SPP.

In vitro anti-proliferative activity of *P. cystidiosus* (non-coremial) and *P. opuntiae* against colon cancer cell line (HeLa) was studied using MTT assay and the results are presented on Table 32. The viability of colon cancer cells treated with the *Pleurotus* spp. extracts was reduced in a concentration dependent manner. Dried extract of *P. cystidiosus* (non-coremial) mushroom at concentrations ranging from 6.25, 12.5, 25, 50 and 100 µg ml^{-1} decreased the viability of colon cancer cells in a concentration dependent manner and recorded per cent viability of 80.89, 71.88, 57.17, 42.18 and 17.77 per cent respectively. Dried extract of *P. opuntiae* mushroom at concentrations ranging from 6.25, 12.5, 25, 50 and 100 µg ml^{-1} recorded decreased per cent viability of colon cancer cell line *viz.*, 88.73, 74.03, 57.25, 46.89 and 29.27 per cent respectively. Dried extract of *P. cystidiosus* (non-coremial) and *P. opuntiae* at concentration of 100 µg ml^{-1} recorded the highest anti-proliferative effect, with minimum cell viability of 17.77 and 29.27 per cent, respectively. The IC-50 values *i.e.* concentration of extract needed for the 50 per cent of cell death for *P. cystidiosus* (non-coremial) and *P. opuntiae* was found as 44.91 and 55.04 µg ml^{-1}. Results indicated that, *P. cystidiosus* (non-coremial) showed significantly more antiproliferative activity than *P. opuntiae* extract, as the former recorded minimum IC 50 value and cancer cell viability.

4.15. KEEPING QUALITY OF *PLEUROTUS* SPP.

Sample of *P. cystidiosus* (non-coremial) could be kept fresh for 48 h under ordinary condition and there after mushrooms started withering. Mushrooms kept under refrigerated conditions (4 ^{0}C) were fresh and white up to 5 days and they were found good for cooking. After six days mushrooms became soft and after ten days, mushrooms lost their freshness and turned creamy white in colour, without

Table 32. Invitro anti-proliferative activity of *Pleurotus* spp. against colon cancer cell line (HeLa)

Treatments	Sample concentration (µg ml^{-1})					IC 50 value (µg ml^{-1})
	*Per cent viability (%)					
	6.25	12.5	25	50	100	
P. opuntiae	88.726	74.029	57.240	46.886	29.270	55.039
P. cystidiosus	80.89	71.88	57.17	42.18	17.77	44.91

*Average of ten replications

LD 50 value of *P. opuntiae* -55.039 µg ml^{-1}

LD 50 value of *P. cystidiosus* -44.91 µg ml^{-1}

off smell and watery appearance. After 15 days mushrooms turned light yellow in colour, with slight watery texture, off smell and slightly sticky. Mushrooms became light brown in colour with slight watery texture, bad smell, more sticky in appearance having small prominent, pin head like growth from edges in 20 days. Per cent reduction in fresh weight for *P. cystidiosus* (non-coremial) was 0.41 per cent, after keeping for 24 h under refrigerated conditions, and showed an increased weight loss, over the days of storage, with maximum per cent reduction at 20 days after storage (39.65 per cent). Hence the results indicated that *P. cystidiosus* (non-coremial) mushrooms could be preserved in refrigerated conditions upto 5 days without any abnormal changes.

Samples of *P. opuntiae, P. florida* and *P. eous* could be kept fresh for 24 h under ordinary condition and there after mushrooms started withering. Mushrooms kept under refrigerated conditions (4 ^{0}C) were fresh, white, without off smell, with watery appearance up to 3 days and they were found good for cooking. After four days mushrooms became soft and the next day, mushrooms lost their freshness and turned creamy white (for *P. opuntiae*) /light yellow (for *P. florida* and *P. eous*) in colour, without off smell and watery appearance. After 10 days, mushrooms turned light brown (for *P. opuntiae*) /yellow (for *P. florida* and *P. eous*) in colour, with slight watery texture, off smell and turned slightly sticky. Mushrooms became dark brown (for *P. opuntiae*) /yellow (for *P. florida* and *P. eous*) in colour with watery texture, bad smell and more sticky in 15 days. *P. opuntiae* recorded 16.12 per cent reduction in fresh weight after keeping for 24 h under refrigerated conditions whereas, *P. florida* and *P. eous* recorded no per cent reduction in fresh weight. *P. opuntiae, P. florida* and *P. eous* showed an increased weight loss over the days with maximum per cent reduction recorded at 15 days after storage *viz*., 74.41, 33.86 and 54.40 per cent respectively. Hence the results indicated that *P. opuntiae*, *P. florida* and *P. eous* mushrooms could be preserved in refrigerated conditions up to three days without any abnormal changes (Table 33 and Plate 39).

Table 33. Keeping quality of *Pleurotus* spp. in refrigerated condition (4 ^{0}C)

Sl. No.	Days after harvest				Observation	*Per cent reduction in fresh weight			
	I	II	III	IV		I	II	III	IV
1	5	3	3	3	Fresh, white, no smell, no watery appearance, good for cooking	8.53	9.84	24.80	13.50
2	6	4	4	4	Fresh, white, no smell, no watery appearance and softened	10.30	13.21	26.71	18.27
3	10	5	5	5	No freshness, creamy white colour, no smell, no watery texture	19.62	15.46	53.05	26.54
4	15	10	10	10	Light yellow colour, slight watery texture, off smell, slightly sticky	29.09	23.75	61.13	36.11
5	20	15	15	15	Light brown colour, slight watery texture, bad smell, more sticky in appearance, small prominent, pinhead like growth started from edges	39.65	33.86	74.41	54.40

I: *P. cystidiosus* non-coremial, II: *P. florida*, III: *P. opuntiae*, IV: *P. eous*

*Average of four replications

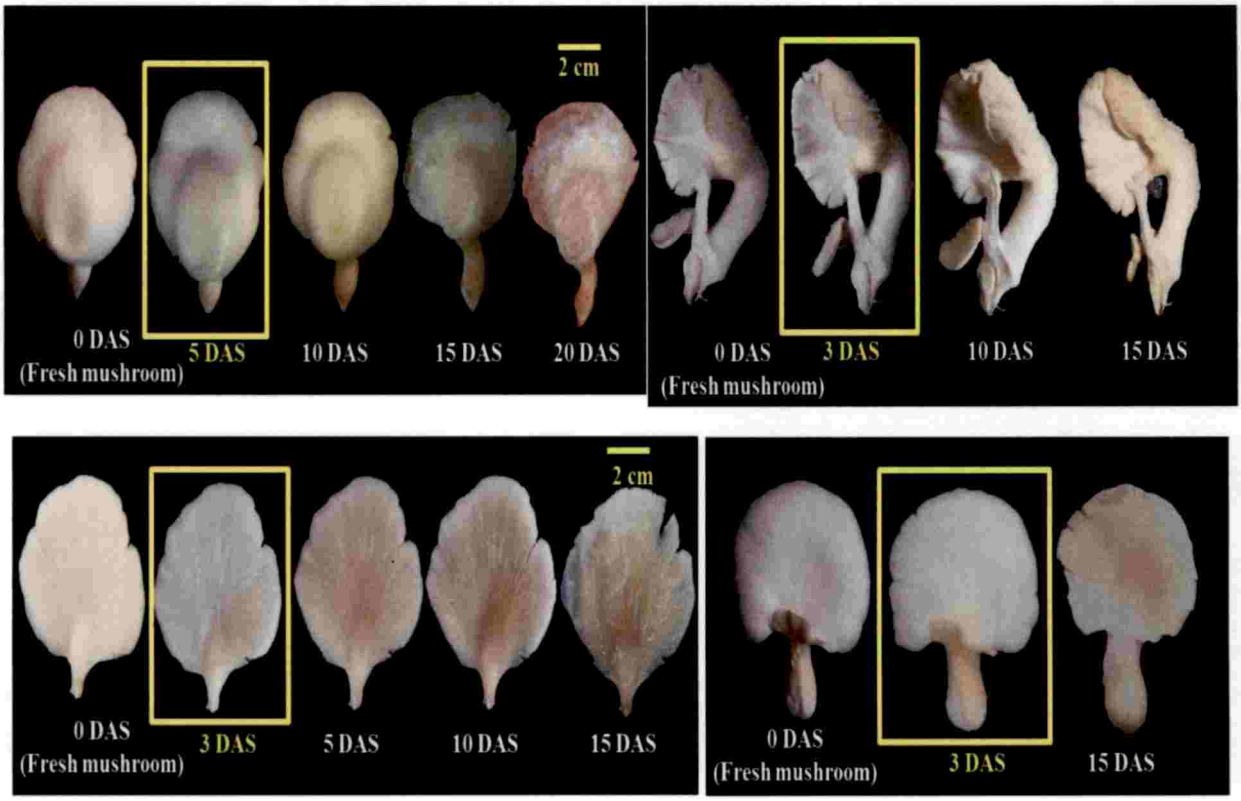


Plate 39. Keeping quality of *Pleurotus* spp. in refrigerated condition (4 ^{0}C)

4.16. SENSORY EVALUATION

Five different recipes were prepared separately using *P. cystidious* (non-coremial), *P. opuntiae*, *P. florida* and *P. eous*, in order to assess their cooking quality. The sensory evaluation was done with 9 point Hedonic rating scale involving evaluation panel of ten members. The recipes prepared for the evaluation were saute, steam cooked mushroom, soup, baji and masala curry (Plate 40).

4.16.1. Sensory evaluation of fruit bodies of four *Pleurotus* spp.

Masala curry prepared from *P. cystidiosus* (non-coremial) recorded the maximum values for appearance (9.1), colour (9.3), flavor (9.0), texture (9.0), taste (9.2), aroma (9.1) and overall acceptability (9.4) among all the recipes of *Pleurotus* spp studied followed by mushroom soup which recorded the maximum acceptability value for *P. cystidious* (non-coremial) (8.4), followed by *P. opuntiae* (8.2), *P. florida* (7.9) and *P. eous* (8.0). Saute, steam cooked mushroom and baji prepared from *P. cystidiosus* (non-coremial) gave the maximum acceptability values of 8.3, 7.7 and 8.2 respectively when compared to the same recipes prepared from the other *Pleurotus* spp. (Table 34).

4.17. POST HARVEST TECHNOLOGY OF PLEUROTUS SPP.

P. cystidiosus (non-coremial) and *P. opuntiae* were subjected to the following post harvest treatments *viz.*, (i) mechanical drying (ii) mechanical drying and powdering (iii) soaking in 1 per cent CA for 15 min followed by mechanical drying (iv) soaking in 1 per cent KMS for 15 min followed by mechanical drying (v) soaking in 1 per cent CA for 15 min followed by mechanical drying and powdering (vi) soaking in 1 per cent KMS for 15 min followed by mechanical drying and powdering (vii) solar drying (control), followed by serial dilution of the samples, at two months interval (up to 6

Table 34. Sensory scores of recipes developed from *Pleurotus* spp.

Sl. No.	Pdts.	*Appearance				*Colour				*Flavour				*Texture				*Taste				*Aroma				*Overall acceptability			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
1	P1	8.0	8.2	8.3	8.0	7.9	8.2	8.4	8.0	7.5	8.2	7.7	7.9	7.7	8.0	7.6	7.5	7.3	8.6	7.5	8.1	8.1	8.4	8.3	8.4	7.7	8.3	7.9	8.0
2	P2	7.6	7.9	8.3	7.7	7.5	7.9	8.3	7.6	7.1	7.7	7.0	7.2	7.5	7.7	7.5	7.5	6.9	7.6	6.8	7.0	7.2	7.4	7.1	7.5	7.3	7.7	7.5	7.4
3	P3	7.9	8.2	9.3	7.9	8.0	8.3	9.3	8.0	7.8	8.1	7.2	7.9	7.7	8.0	7.4	7.6	7.7	8.9	7.1	8.8	8.4	8.7	8.0	8.7	7.9	8.4	8.0	8.2
4	P4	7.8	7.9	7.8	7.8	7.9	8.0	7.9	7.9	7.7	7.9	7.5	7.8	7.6	7.8	7.5	7.6	7.5	9.0	7.4	8.5	8.6	8.8	8.5	8.9	7.8	8.2	7.8	8.1
5	P5	8.9	9.1	9.0	8.8	8.8	9.0	8.9	8.8	7.9	9.0	7.8	8.6	8.7	9.0	8.6	8.6	7.6	9.2	7.6	8.7	8.8	9.0	8.7	9.1	8.5	9.4	8.4	8.8

*Average of ten replications (0-5 scores), I- *P. florida*, II- *P. cystidiosus* non-coremial, III- *P. eous*, IV- *P. opuntiae*, P1: Saute, P2: Steam cooked, P3: Soup, P4: Baji, P5: Masala curry

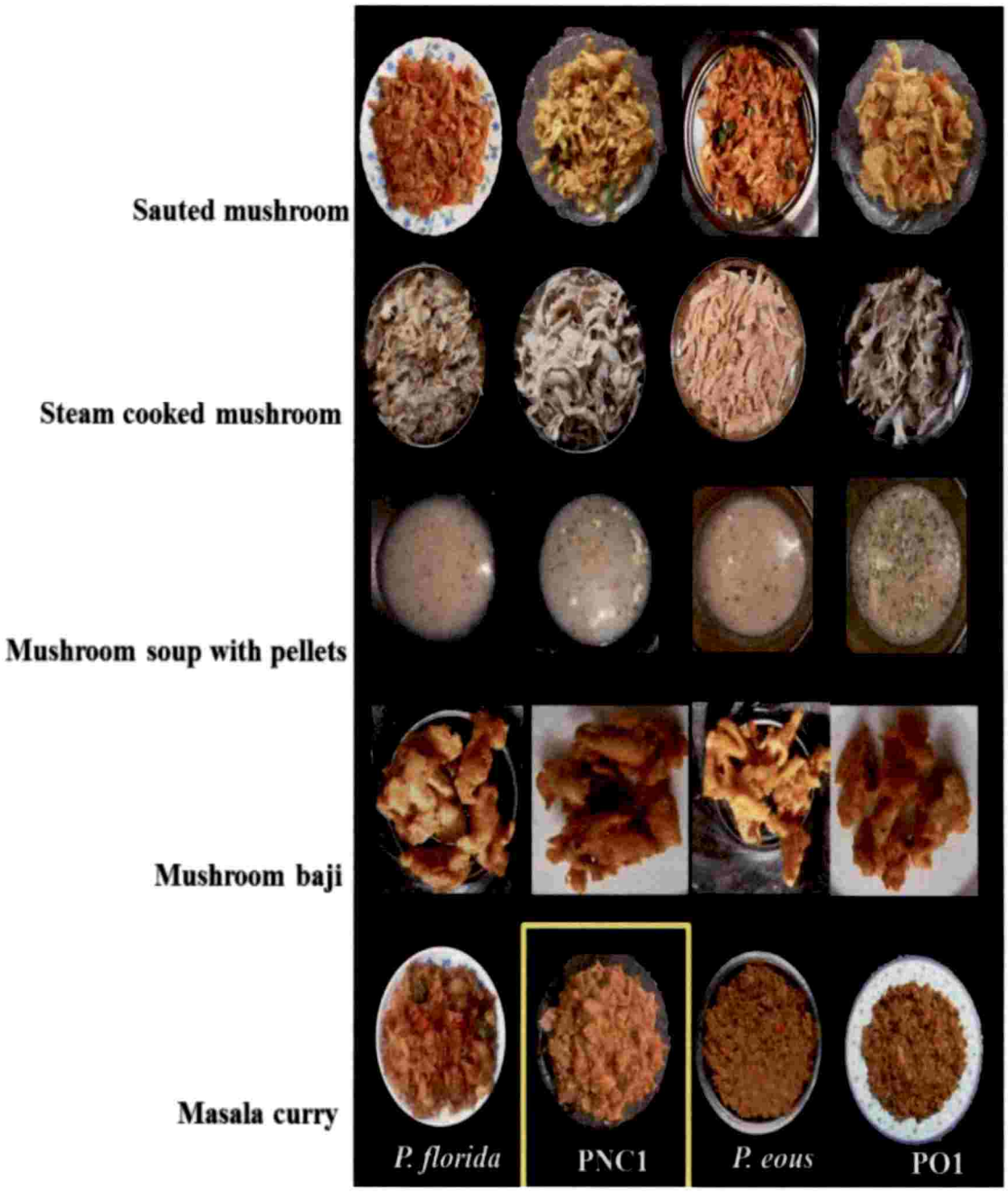


Plate 40. Mushroom recipes of *Pleurotus* spp.

Table 35. Population dynamics of saprophytic fungi and bacteria in *Pleurotus* spp. samples subjected to post harvest treatments at 2 months interval.

Sl. No.	Trts	*P. cystidiosus* non-coremial		*P. opuntiae*	
		Saprophytic fungi (cfu/g x 10^4)*	Saprophytic bacteria (cfu/g x 10^6)*	Saprophytic fungi (cfu/g x 10^4)*	Saprophytic bacteria (cfu/g x 10^6)*
		7 MAT	7 MAT	5 MAT	5 MAT
1	T1	0^{d} (0.701)	1.00^{d} (1.23) (*Bacillus* sp.)	0^{c} (0.701)	7.67^{c} (2.77) (*Bacillus* sp.)
2	T2	0^{d} (0.701)	5.33^{b} (2.41) (*Bacillus* sp.)	0^{c} (0.701)	8.33^{c} (2.89) (*Bacillus* sp.)
3	T3	1.67^{c} (1.46) (*Aspergillus* sp.)	0^{e} (0.701)	3.33^{b} (1.953) (*Penicillium* sp.)	19.00^{b} (4.36) (*Bacillus* sp.)
4	T4	0^{d} (0.701)	1.00^{d} (1.23) (*Bacillus* sp.)	0^{c} (0.701)	1.33^{e} (1.13) (*Bacillus* sp.)
5	T5	3.33^{a} (1.95) (*Trichoderma* sp.)	0^{e} (0.701)	0^{c} (0.701)	1.67^{de} (1.27) (*Bacillus* sp.)
6	T6	0^{d} (0.701)	2.33^{c} (1.68) (*Bacillus* sp.)	0^{c} (0.701)	2.33^{d} (1.52) (*Bacillus* sp.)
7	T7	2.33^{b} (1.68) (*Trichoderma* sp.)	49.67^{a} (7.09) (*Bacillus* sp.)	14.67^{a} (3.90) (*Rhizopus* sp., *Aspergillus* sp., *Trichoderma* sp.)	40.33^{a} (6.36) (*Bacillus* sp.)
SE m (±)		0.526	6.901	2.070	5.337
CD (0.05)		0.199	0.131	0.107	0.281

MAT: Months after treatment

T1: Citric acid treatment (1 per cent) + mechanical drying, T2: KMS (1 per cent) + mechanical drying, T3: Mechanical drying, T4: T1+powdering, T5: T2+powdering, T6: T3+powdering, T7: Solar drying (Control).

*Average of three replications, Means followed by similar superscripts are not significantly different at 5 % level, Figures in parenthesis are transformed values

months), to quantify the associated microflora *viz*., fungi and bacteria. The results of study are presented in Table 35 and Plate 41.

Samples of *P. cystidiosus* (non-coremial) mushrooms subjected to the different post harvest treatments can be stored up to 6 months without any contamination. Serial dilution was done 7 months after post harvest treatment which recorded minimum contamination in CA treatment (1 per cent)+mechanical drying (1 bacterial cfu g^{-1} $x10^6$) and CA treatment (1 per cent)+mechanical drying+powdering (1 bacterial cfu g^{-1} $x10^6$), followed by mechanical drying (1.67 fungal cfu g^{-1} $x10^6$) and mechanical drying+powdering (2.33 bacterial cfu/g $x10^6$). *Bacillus* sp., *Aspergillus* sp and *Trichoderma* sp. were the microflora associated with the treated mushrooms. In case of *P. cystidiosus* (non-coremial), citric acid treatment (1 per cent)+mechanical drying and citric acid treatment (1 per cent)+mechanical drying+powdering were the best post harvest treatments followed by mechanical drying and mechanical drying+powdering; and these treated mushrooms can be stored free of contamination up to 6 months.

Samples of *P. opuntiae* mushrooms subjected to the different post harvest treatments can be stored up to 4 months, without any contamination. Serial dilution was done 5 months after post harvest treatment which recorded minimum contamination in citric acid treatment (1 per cent)+mechanical drying+powdering (1.33 bacterial cfu g^{-1} $x10^6$) followed by KMS (1 per cent)+mechanical drying+powdering (1.67 bacterial cfu g^{-1} $x10^6$) and mechanical drying+powdering (2.33 bacterial cfu g^{-1} $x10^6$). *Bacillus* sp., *Aspergillus* sp and *Trichoderma* sp., *Rhizopus* sp. and *Penicillium* sp. were the microflora associated with the treated samples. Thus in case of *P. opuntiae* CA treatment (1 per cent)+mechanical drying+powdering was the best post harvest treatments followed by KMS (1 per cent)+mechanical drying+powdering and mechanical drying+powdering; and the mushrooms can be stored up to 4 months.

4.18. STRAIN IMPROVEMENT OF *PLEUROTUS* SPP.

4.18.1. Barrage reaction

Compatibility studies revealed a positive mating reaction between the crosses *viz.*, *P. cystidiosus* (coremial) x *P. opuntiae* and *P. cystidiosus* (non-coremial) x *P. opuntiae*, indicated by the formation of a thick barrage of intermingled hyphae at the zone of contact (Plate 42).

4.18.2. Biochemical markers

P. opuntiae took 7, 7.33 and 7.67 days for completion of growth in petridishes amended with carbendazim at 0.1, 0.5 and 1 m*M* while no growth was recorded with vanillin at 0.05 per cent concentration. *P. cystidiosus* (coremial) showed absence of growth in carbendazim, at all three concentrations and took 26.67 days for completion of growth in petridish amended with vanillin at 0.01 per cent concentration. Hence, no dual biochemical markers could be identified for *P. opuntiae* and *P. cystidiosus* (coremial). *P. cystidiosus* (non-coremial) showed absence of growth in carbendazim, at all three concentrations, while it took 6.90, 8.00 and 8.67 days for completion of growth in petridishes amended with, vanillin 0.01, 0.02 and 0.05 per cent, respectively. In the present experiment among the parent strains, *P. opuntiae* was insensitive to the fungicide carbendazim, at all three concentrations, but it couldnt utilize vanillin (0.05 per cent), while *P. cystidiosus* (non-coremial) was sensitive to carbendazim, at all three concentrations but could utilise vanillin, at all three concentrations. Thus, in PDA plates amended with both carbendazim (1 m*M*) and vanillin (0.05 per cent), the growth of both parents was inhibited. But the fusant showed uniform growth rate in all the plate assays indicating that it had acquired the characters of both the parents. As it showed biparental morphology, it could be confirmed as a recombinant (Table 36 and Plate 43).

4.18.3. Protoplast isolation

Release of protoplasts depend on many factors *viz.*, age of mycelium, enzymes and osmotic stabilisers used; and time of incubation. Results of the study are presented in Table 37, 38 & 39.

Table 36. Selection of dual chemical markers for protoplast fusants

Sl. No.	Biochemical markers	*P. opuntiae* *		*P. cystidiosus* (coremial)*		*P. cystidiosus* (non-coremial)*	
1	Carbendazim 0.1 m*M*	7.00	+	-	-	-	-
2	Carbendazim 0.5 m*M*	7.33	+	-	-	-	-
3	Carbendazim 1 m*M*	7.67	+	-	-	-	-
4	Vanillin 0.01 %	9.33	+	26.67	+	6.90	+
5	Vanillin 0.02 %	12.33	+	-	-	8.00	+
6	Vanillin 0.05 %	-	-	-	-	8.67	+
7	Control	7.00	+	24.00	+	7.33	+

*Average of five replications

+: Presence of growth

-: Absence of growth

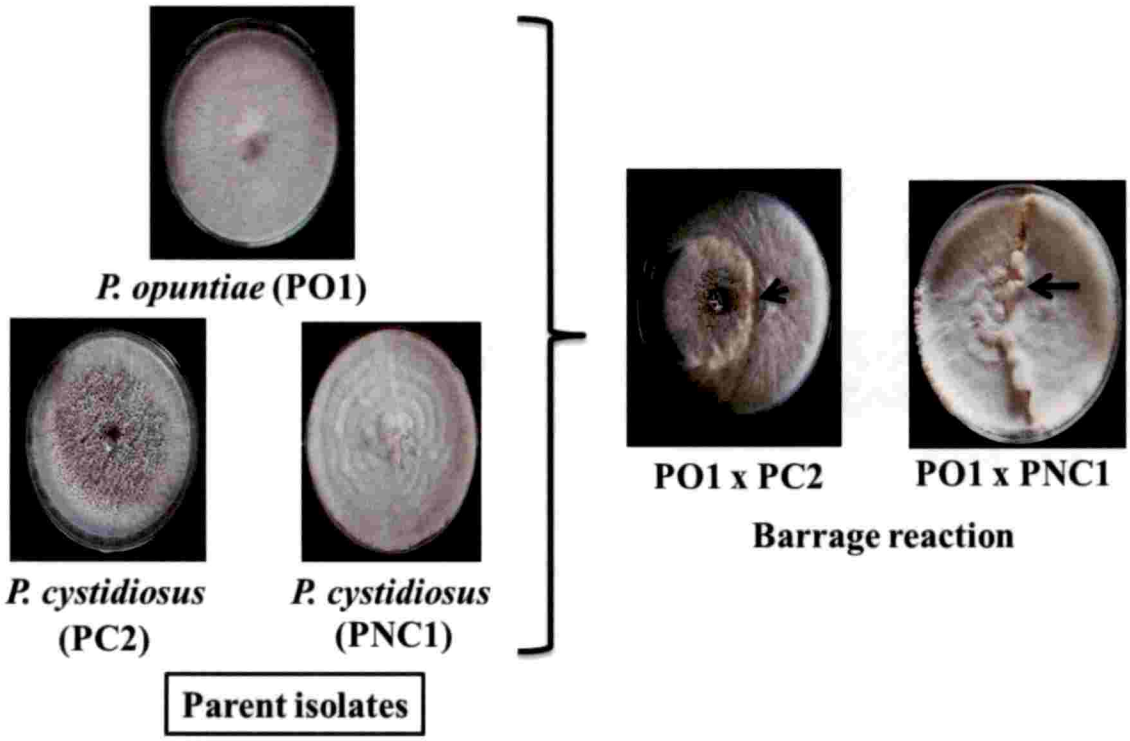


Plate 42. Compatibility studies between *Pleurotus* spp.

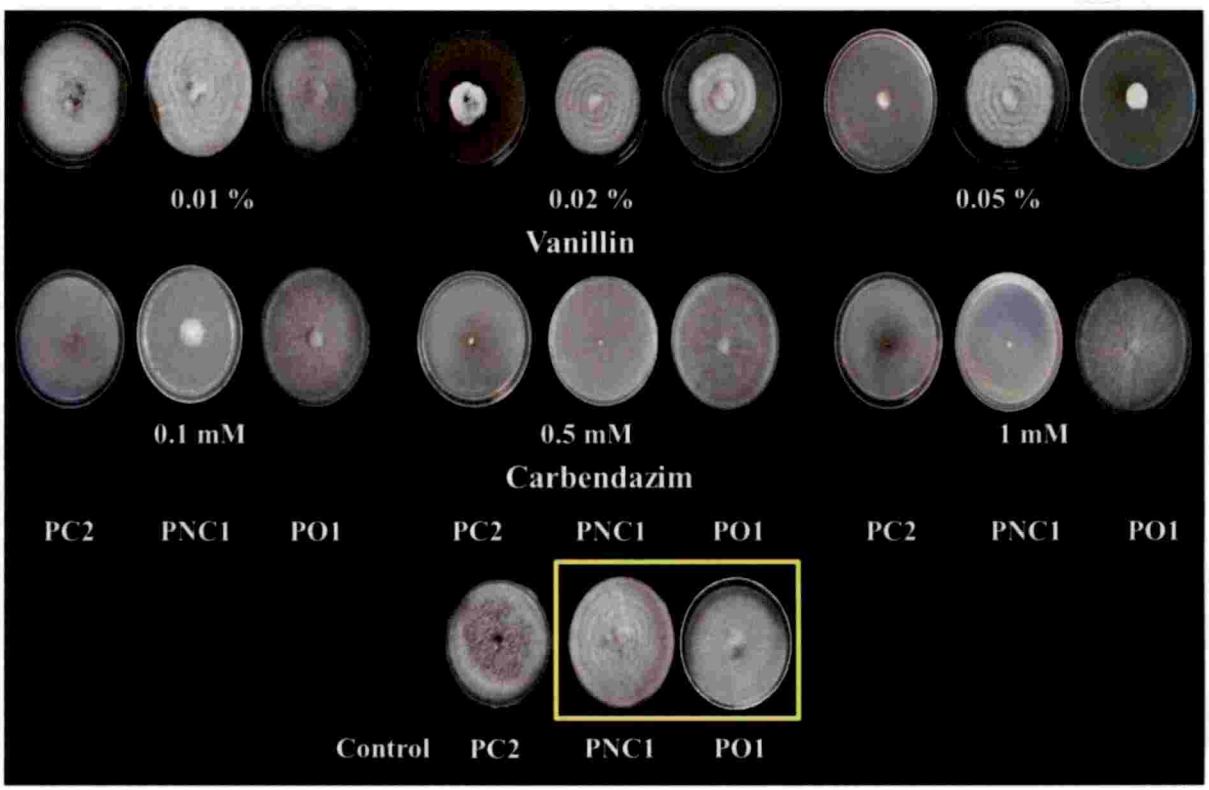


Plate 43. Selection of chemical markers for protoplast fusants

Table 37. Effect of enzyme concentration, osmoticum and time of incubation on the protoplast yield ($x10^7$cells ml^{-1}) of parental strains at 3 days age of mycelium.

Incubation time (h)	*Protoplasts of *P. opuntiae* ($x10^7$cells ml^{-1})						*Protoplasts of *P. cystidiosus* (non-coremial) ($x10^7$cells ml^{-1})					
	Osmoticum type-0.6 *M* KCl			Osmoticum type-0.6 *M* sucrose			Osmoticum type-0.6 *M* KCl			Osmoticum type- 0.6 *M* sucrose		
	Enzyme concentration (mg ml^{-1})			Enzyme concentration (mg ml^{-1}l)			Enzyme concentration (mg ml^{-1})			Enzyme concentration (mg ml^{-1})		
	20	25	30	20	25	30	20	25	30	20	25	30
1	0^g (0.70)	0^g (0.701)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)
2	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)
3	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^g (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)
3.5	2.91^c (1.84)	3.10^c (1.91)	3.44^c (1.98)	0.91^d (1.18)	0.95^d (1.20)	1.13^d (1.28)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)
4	3.23^a (1.94)	4.70^a (2.29)	7.87^a (2.90)	1.61^a (1.45)	1.89^a (1.55)	4.10^a (2.15)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)
4.5	3.00^b (1.88)	3.62^b (2.02)	6.64^b (2.68)	1.30^b (1.34)	1.48^b (1.41)	3.47^b (1.99)	1.56^c (1.43)	1.63^c (1.47)	1.70^c (1.49)	1.00^c (1.23)	1.06^c (1.25)	1.10^c (1.27)
5	0.90^d (1.18)	1.08^d (1.260)	3.21^d (1.92)	0.93^c (1.20)	0.98^c (1.22)	1.22^c (1.31)	2.32^a (1.68)	3.10^a (1.91)	5.93^a (2.54)	1.10^a (1.27)	1.20^a (1.31)	4.23^a (2.17)
5.5	0.84^e (1.15)	0.97^e (1.21)	1.10^e (1.28)	0.90^e (1.18)	0.94^e (1.20)	0.94^e (1.20)	2.20^b (1.63)	2.60^b (1.77)	4.76^b (2.29)	1.06^b (1.26)	1.18^b (1.29)	3.40^b (1.97)
6	0.75^f (1.11)	0.88^f (1.180)	0.94^f (1.20)	0.81^f (1.15)	0.86^f (1.16)	0.87^f (1.18)	0.91^d (1.19)	0.96^d (1.21)	1.67^d (1.48)	0.87^d (1.18)	0.89^d (1.18)	0.92^d (1.20)
SE m (±)	0.456	0.587	0.989	0.197	0.225	0.499	0.334	0.413	0.760	0.178	0.192	0.542
CD (0.05)	0.008	0.004	0.005	0.001	0.005	0.008	0.006	0.007	0.004	0.003	0.003	0.009

*Average of four replications, Means followed by similar superscripts are not significantly different at 5 per cent level, Figures in the parenthesis are transformed values

Table 38. Effect of enzyme concentration, osmoticum and time of incubation on the protoplast yield ($x10^7$ cells ml^{-1}) of parental strains at 4 days age of mycelium.

Incubation time (h)	*Protoplasts of *P. opuntiae* ($x10^7$cells ml^{-1})						*Protoplasts of *P. cystidiosus* (non-coremial) ($x10^7$cells ml^{-1})					
	Osmoticum type- 0.6 *M* KCl			Osmoticum type- 0.6 *M* sucrose			Osmoticum type-0.6 *M* KCl			Osmoticum type-0.6 *M* sucrose		
	Enzyme concentration (mg ml^{-1})			Enzyme concentration (mg ml^{-1})			Enzyme concentration (mg ml^{-1})			Enzyme concentration (mg ml^{-1})		
	20	25	30	20	25	30	20	25	30	20	25	30
1	0^g (0.70)	0^f (0.70)	0^g (0.70)	0^g (0.70)	0^f (0.70)	0^f (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^d (0.70)	0^e (0.70)
2	0^g (0.70)	0^f (0.70)	0^g (0.70)	0^g (0.70)	0^f (0.70)	0^f (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^d (0.70)	0^e (0.70)
3	0^g (0.70)	0^f (0.70)	0^g (0.70)	0^g (0.70)	0^f (0.70)	0^f (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^d (0.70)	0^e (0.70)
3.5	1.20^c (1.31)	1.40^c (1.38)	1.70^d (1.49)	0.88^e (1.18)	0.90^d (1.18)	1.01^c (1.24)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^d (0.70)	0^e (0.70)
4	2.65^a (1.77)	3.80^a (2.08)	5.85^a (2.52)	1.40^a (1.38)	1.42^a (1.39)	3.10^a (1.89)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^e (0.70)	0^d (0.70)	0^e (0.70)
4.5	2.40^b (1.71)	3.52^b (2.01)	5.20^b (2.38)	1.29^b (1.34)	1.39^b (1.38)	3.10^a (1.89)	1.16^c (1.28)	1.20^c (1.33)	1.25^c (1.33)	0.82^c (1.15)	0.86^b (1.16)	0.88^c (1.18)
5	1.12^d (1.28)	1.30^d (1.34)	2.52^c (1.73)	0.90^c (1.18)	0.94^c (1.20)	1.10^b (1.28)	2.10^a (1.63)	2.90^a (1.85)	5.76^a (2.51)	1.00^a (1.25)	1.10^a (1.27)	4.10^a (2.15)
5.5	0.89^e (1.18)	0.95^e (1.20)	1.51^e (1.42)	0.89^d (1.18)	0.91^d (1.19)	0.91^d (1.18)	2.00^b (1.61)	2.40^b (1.71)	4.14^b (2.16)	0.88^b (1.18)	1.07^a (1.26)	3.00^b (1.89)
6	0.80^f (1.14)	0.91^f (1.19)	1.30^f (1.34)	0.79^f (1.14)	0.82^e (1.15)	0.83^e (1.15)	0.88^d (1.18)	0.93^d (1.20)	1.20^d (1.31)	0.75^d (1.12)	0.76^c (1.13)	0.76^d (1.13)
SE m (±)	0.329	0.479	0.728	0.183	0.191	0.404	0.297	0.379	0.711	0.153	0.170	0.509
CD (0.05)	0.006	0.002	0.006	0.001	0.009	0.006	0.017	0.013	0.009	0.014	0.009	0.019

*Average of four replications

Means followed by similar superscripts are not significantly different at 5 per cent level, Figures in the parenthesis are transformed values

Table 39. Effect of enzyme concentration, osmoticum and time of incubation on the protoplast yield ($x10^7$ cells ml^{-1}) of parental strains at 5 days age of mycelium.

Incubation time (h)	*Protoplasts of *P. opuntiae* ($x10^7$cells ml^{-1})						*Protoplasts of *P. cystidiosus* (non-coremial) ($x10^7$cells ml^{-1})					
	Enzyme concentration (mg ml^{-1})						Enzyme concentration (mg ml^{-1})					
	Osmoticum type- 0.6 *M* KCl			Osmoticum type- 0.6 *M* sucrose			Osmoticum type- 0.6 *M* KCl			Osmoticum type- 0.6 *M* sucrose		
	20	25	30	20	25	30	20	25	30	20	25	30
1	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{f} (0.70)	0^{e} (0.70)	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)
2	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{f} (0.70)	0^{e} (0.70)	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)
3	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{f} (0.70)	0^{e} (0.70)	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)
3.5	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{f} (0.70)	0^{e} (0.70)	0^{d} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)
4	0.88^{c} (1.18)	0.91^{d} 1.19	1.01^{d} (1.01)	0.86^{e} (1.16)	0.88^{d} (1.18)	0.89^{c} (1.18)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)	0^{e} (0.70)
4.5	1.35^{a} (1.37)	1.57^{a} (1.44)	2.89^{a} (2.90)	1.39^{a} (1.38)	1.41^{a} (1.39)	3.10^{a} (1.89)	0.80^{d} (0.813)	0.81^{c} (1.15)	0.85^{c} (1.16)	0.77^{c} (1.12)	0.79^{c} (1.14)	0.80^{c} (1.14)
5	1.33^{a} (1.35)	1.50^{b} (1.42)	2.33^{b} (2.34)	1.28^{b} (1.34)	1.37^{b} (1.36)	3.09^{a} (1.89)	1.60^{a} (1.455)	1.65^{a} (1.47)	2.77^{a} (1.81)	0.98^{a} (1.22)	1.00^{a} (1.25)	4.00^{a} (2.15)
5.5	1.0^{b} (1.27)	1.0^{c} (1.27)	1.10^{c} (1.15)	0.89^{c} (1.18)	0.91^{c} (1.18)	0.99^{b} (1.23)	0.91^{b} (1.184)	0.97^{b} (1.21)	1.80^{b} (1.52)	0.87^{b} (1.18)	0.90^{b} (1.19)	2.90^{b} (1.85)
6	0.88^{c} (1.18)	0.90^{d} (1.18)	0.99^{d} (0.10)	0.87^{d} (1.18)	0.91^{c} (1.18)	0.91^{c} (1.18)	0.61^{c} (1.059)	0.67^{d} (1.08)	0.70^{d} (1.10)	0.69^{d} (1.09)	0.70^{d} (1.10)	0.70^{d} (1.10)
SE m (±)	0.199	0.221	0.359	0.195	0.203	0.422	0.193	0.201	0.332	0.148	0.151	0.496
CD (0.05)	0.019	0.018	0.030	0.004	0.009	0.009	0.107	0.002	0.002	0.006	0.013	0.010

*Average of four replications

Means followed by similar superscripts are not significantly different at 5 per cent level, Figures in the parenthesis are transformed values

4.18.3.1. Effect of Mycelium Age on Protoplasts Formation

Maximum release of protoplasts from *P. opuntiae*, was recorded from 3 days old mycelium (7.87 x 10^7 cells ml^{-1}) followed by 4 days (5.85 x 10^7 cells ml^{-1}) aged mycelium, when 0.6 *M* KCl was used as osmotic stabiliser, at 4 hrs after incubation with 30 mg ml^{-1} of enzyme concentration. However protoplasts of *P. opuntiae*, showed regeneration through budding, when 3 days old mycelium was used. Hence, the age of mycelium was optimised as 4 days old. Initially, the protoplasts gave rise to a chain of yeast-like cells which arose from a single growth point. As the regeneration proceeded, chain of cells lengthened and up to 20 cells were produced. Eventually the terminal cell produced a hypha and the newly formed hypha lengthened, branched and ultimately gave rise to a new mycelium. A proportional decrease in the size of protoplasts was also recorded with increasing age of mycelium, *i.e.* 5.98 µm x 5.61 µm (at 3 days old mycelium) to 2.40 µm x1.65 µm (4 days old), with increasing granular residual hyphae and cell debris (Plates 44 & 45).

4.18.3.2. Effects of Enzymes on Protoplast Isolation

P. opuntiae recorded an increased release of protoplasts, with increased concentration of enzyme from 20 to 30 mg/ml for all treatments with 0.6 *M* KCl, *i.e.* 3.23, 4.70 and 7.87 x10^7 cells ml^{-1} at 20, 25 and 30 mg ml^{-1} of enzyme concentration, respectively, at 4 hrs of incubation of 3 days old mycelium. Protoplast yield from 4 days old mycelium, with 0.6 *M* KCl was recorded as 2.65, 3.80 and 5.85 x 10^7 cells ml^{-1} at 20, 25 and 30 mg ml^{-1} of enzyme concentration respectively. Similarly, *P. opuntiae* recorded increased protoplast release from 3 days old mycelium with increasing enzyme concentration with 0.6 *M* sucrose *i.e*, 1.61 at 20, 1.89 at 25 and 4.10 at 30 mg ml^{-1} at 4 h of incubation. Four days old mycelium recorded 1.40, 1.42 and 3.10x10^7 protoplasts ml^{-1} at 20, 25 and 30 mg/ml of enzyme concentration respectively with 0.6 *M* sucrose. At low concentration of enzyme (15 mg ml^{-1}), the lysis of fungal mycelium was confined only to a small portion whereas at high enzyme concentrations (35 mg ml^{-1}), the

mycelium lysed effectively yielding large numbers of immature protoplasts, which bursted immediately after release and got disintegrated (Plate 44 & 45).

4.18.3.3. Effect of Osmotic Stabilizers

Two osmotic stabilisers were tried *viz.*, 0.6 *M* KCl and 0.6 *M* sucrose at different enzyme concentration and time intervals. 0.6 *M* KCl yielded the maximum protoplasts, at 4 h of incubation of 3 days old mycelium (7.87×10^7 cells/ml) followed by 0.6 *M* KCl at 4 h of incubation of 4 days old mycelium (5.85×10^7 cells ml^{-1}) and 0.6 *M* sucrose, at 4 h incubation of 3 days old mycelium (4.10×10^7 cells ml^{-1}).

4.18.3.4. Time of incubation

A constant gradation was observed with increasing time of incubation, *i.e.* protoplast released increased and reached a peak at a particular time interval and showed a decline thereafter. Mycelium of *P. opuntiae* at 3 and 4 days old recorded no protoplast release up to 3 h with either 0.6 *M* KCl or 0.6 *M* sucrose followed by a maximum release at 4 h of incubation and a gradual decrease thereafter. However *P. opuntiae* at 5 days old recorded no protoplast release up to 3.5 hours and maximum release was recorded at 4.5 h of incubation followed by a gradual decline (Plate 44 & 45). Thus, an optimized protocol involving 5 h hydrolysis of 3 day old mycelium of *P. cystidiosus* (non-coremial) and 4 h hydrolysis of 4 day old mycelium of *P. opuntiae* was standardised using 30 mg ml^{-1} of commercially available lysing enzyme from *Trichoderma harzianum* in a 1 : 1 (w/w) biomass : enzyme ratio, with 0.6 *M* KCl as osmotic stabilizer (Table 40).

At the optimised age of *Pleurotus spp.* initial lysis of the protoplast was observed 2 h after enzyme treatment, followed by swelling and rounding up of cell content within 3 h. Complete lysis of mycelium and release of protoplasts from mycelial tips was observed at 3.5 and 4.5 h for *P. opuntiae* and *P. cystidiosus* (non-coremial) respectively. Maximum release of protoplast was

recorded at 4 and 5 h for *P. opuntiae* and *P. cystidiosus* (non-coremial) respectively. Also bursting and considerable decrease in protoplasts was recorded 5 and 6 h after enzyme incubation respectively. Micrographs showed that mycelium of *Pleurotus* spp. was first subdivided into small irregular fragments and there was some degree of cytoplasmic shrinkage at the beginning of incubation. Then the fragments were transformed into distorted cells and at the end of the incubation almost all the cells were converted into protoplasts. At early stages protoplasts were mostly emerging from the hyphal tips but, as cell wall degradation progressed, protoplasts emerged from other regions in addition to the tips. The protoplasts released from *Pleurotus* spp. initially were smaller in size but later they enlarged to spherical structures.

4.18.4. Regeneration of protoplasts

Regeneration of protoplasts refers to reversion to mycelial form and is the starting point for downstream genetic manipulation. MYG with the standardised osmotic stabilizer *i.e*, 0.6 *M* KCl (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) was used as regeneration medium, at 24 ^{0}C for the protoplasts to develop into small colonies and further to normal morphology. After 48 h of incubation, microcolonies were seen and was observed under stereo microscope. *P. cystidiosus* (non-coremial) recorded significantly more number of colonies (3200.80), compared to *P. opuntiae* (2220.00), with fusion frequencies of 0.54 and 0.38 respectively (Table 41). Regenerated colonies were separately, transferred to PDPA. The microcolonies arosed from the regenerated protoplasts. The germinating protoplasts soon developed into filamentous mycelia and exhibited no variation in mycelial morphology from the parent.

4.18.5. PEG mediated fusion and regeneration

Protoplasts of *P. cystidiosus* (non-coremial) and *P. opuntiae* at the optimized condition, recorded sizes of 1.50 µm x 1.35 µm size and 2.40 µm x1.65

Table 40. Protoplast yields using optimised protocols

Sl. No.	Parents	Age of mycelium (days)	Incubation time (days)	Concentration of enzyme (mg ml^{-1})	Osmoticant	Time of maximum release of protoplasts (hours)	Protoplasts ml^{-1}
1	Parent 1	4	4	30	0.6 *M* KCl	4	5.85×10^{7}
2	Parent 2	3	3	30	0.6 *M* KCl	5	5.93×10^{7}

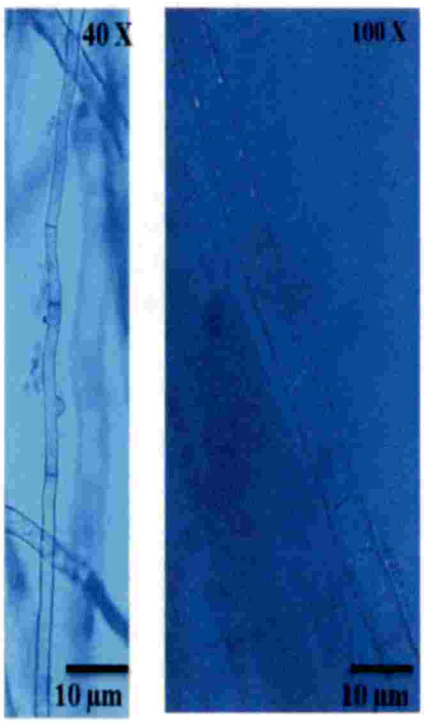
Parent 1: *P. opuntiae*, Parent 2: *P. cystidiosus* (non-coremial)

Table 41. Regeneration frequency of protoplast isolates and protoplast fusants on regeneration medium (Malt-yeast extract glucose medium)

Sl. No.	Protoplast isolates/fusants	Number of colonies regenerated on RM*	
		Counts	Regeneration/Fusion frequency
1	POi	2220.00^{b}	0.38^{b}
2	PNCi	3200.80^{a}	0.54^{a}
3	Fusants	1270.60^{c}	0.21^{c}
SE m (±)		557.22	0.095
CD (0.05)		0.974	0.001

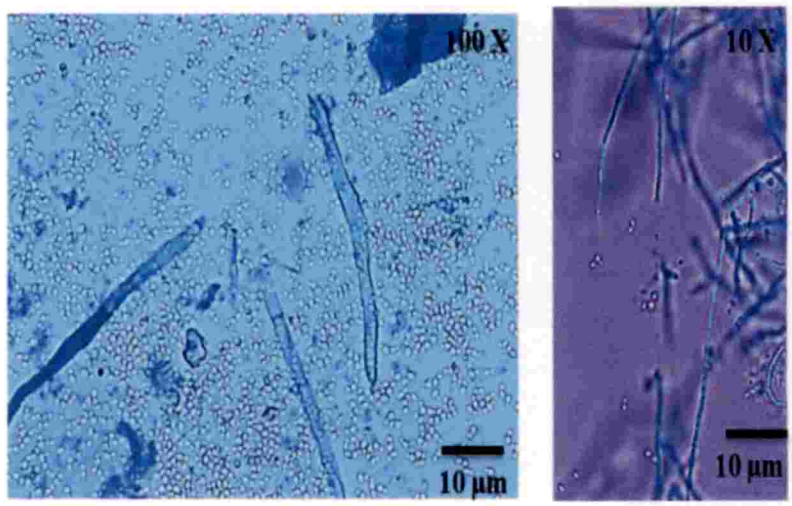
*Average of five replications, Means followed by similar superscripts are not significantly different at 5% level

POi: protoplast isolate of *P. opuntiae*, PNCi: protoplast isolate of *P. cystidiosus* (non-coremial)

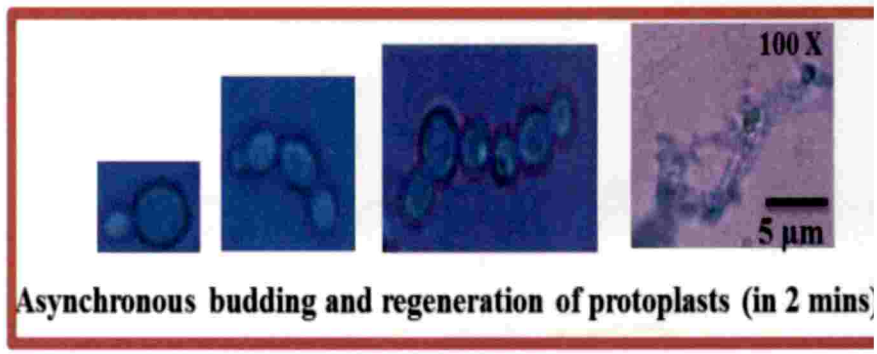


Before treatment with enzyme

Mycelium subdivided to distorted cells 2 h



Protoplasts released from hyphae tips 4 h



Asynchronous budding and regeneration of protoplasts (in 2 mins)

Plate 44. Stages of release of protoplasts from 4 days old *P. opuntiae* with 0.6 *M* KCl and 30 mg ml $^{-1}$ of enzyme

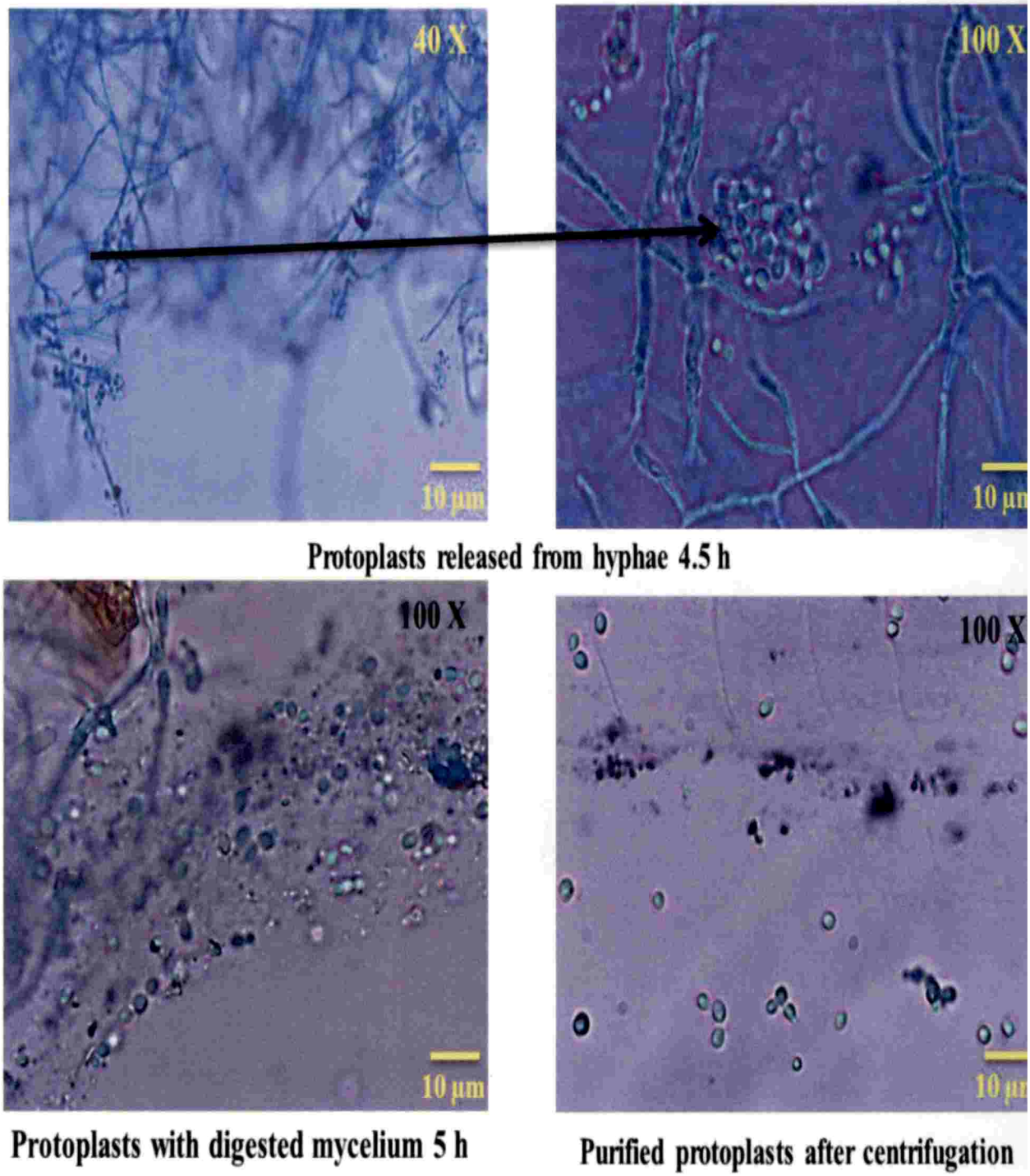


Plate 45. Stages of release of protoplasts from 3 days old *P. cystidiosus* (non-coremial), with 0.6 *M* KCl and 30 mg ml $^{-1}$ of enzyme

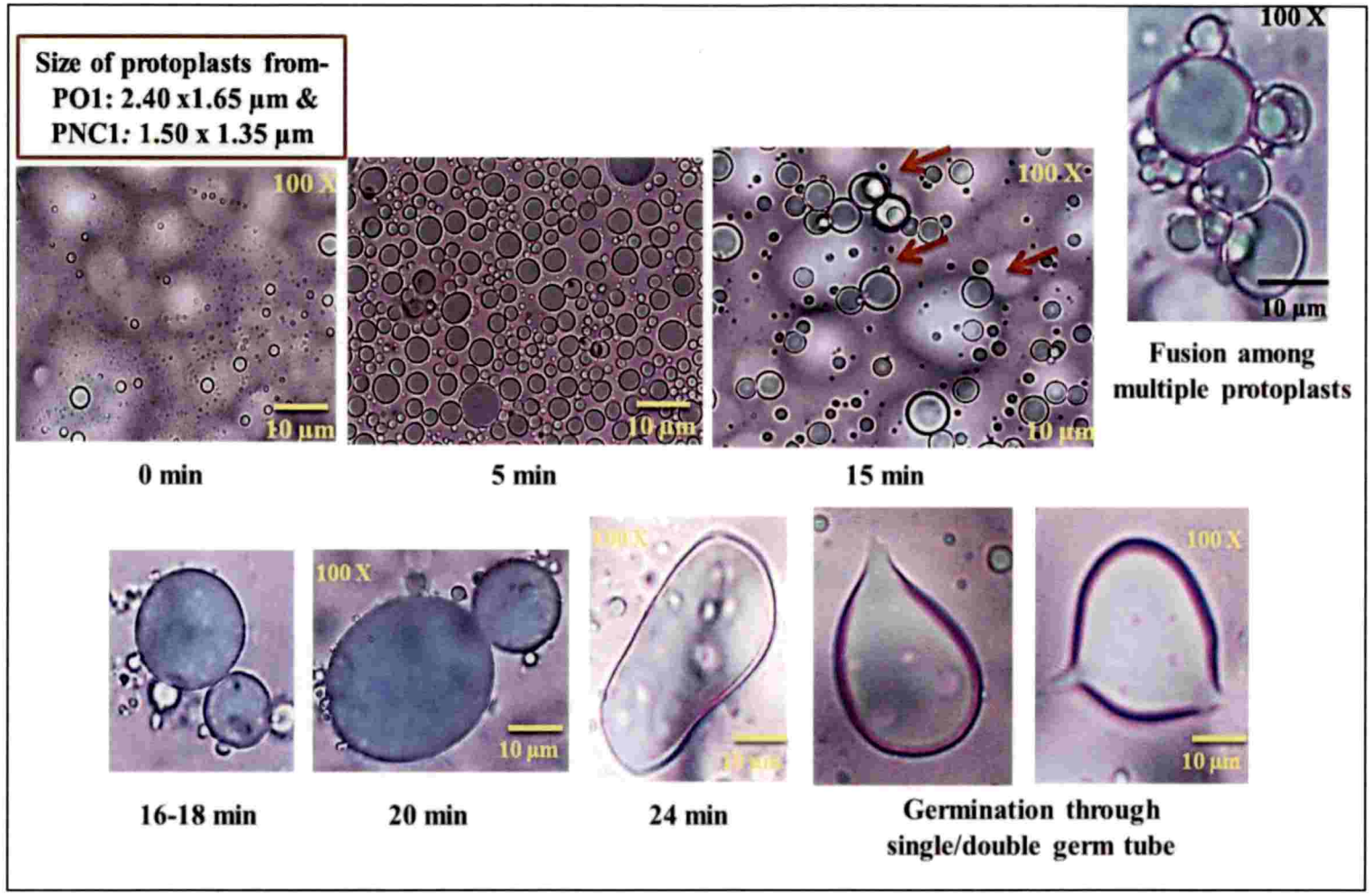
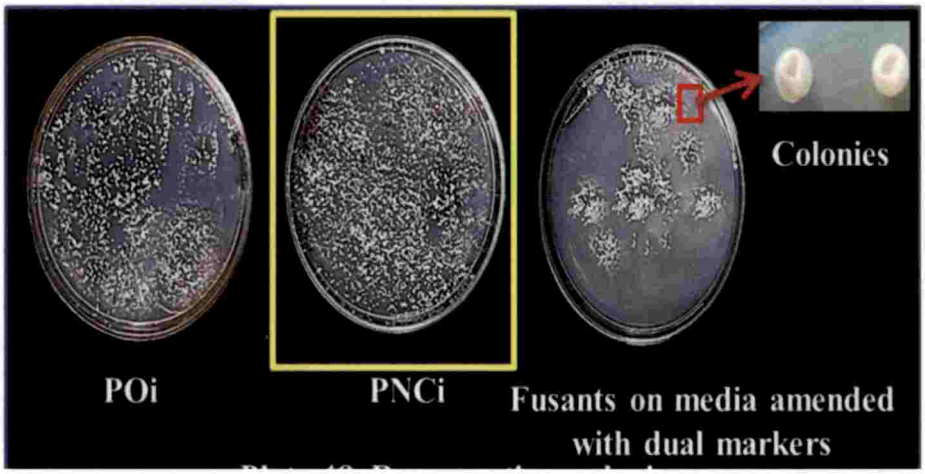
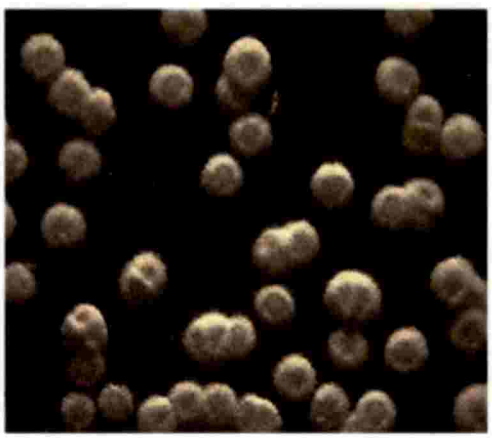


Plate 46. Fusion process between *Pleurotus* spp.





Colonies under stereo microscope

Plate 47. Regeneration of colonies

μm size respectively. With the addition of PEG solution, their sizes increased to 2.14 μm x 1.50 μm and 3.07 μm x 2.71 μm, respectively in 5 minutes. Protoplasts were attracted and adhered to each other in 15 minutes, accompanied by increase in sizes to 2.84 μm x 1.99 μm and 7.92 μm x 6.72 μm respectively. In next 5 min, size increased from 13.06 μm x 11.18 μm and 43.73 μm x 31.07 μm to 55.66 μm x 53.56 μm and 102.37 μm x 113.72 μm respectively, followed by their fusion to form an elongated, oval cell of size 24.68 μm x 11.49 μm. During the next 4 min germination was observed through formation of one or two germ tubes. Self-fusion and multiple of protoplasts was also observed (Plate 46). The fused protoplasts were plated on MYG medium with biochemical markers *viz*., vanillin 0.05 per cent and carbendazim 1 m*M* for selection of putative fusants and avoiding self fusants. They started regenerating in two days followed by development of mycelium in three days. Spherical fused protoplasts regenerated directly by producing one or more germ tubes (Plate 47).

4.18.6. Confirmation and evaluation of strains/hybrids

4.18.6.1. Selection on the Basis of Radial Growth

Eight protoplast fusant lines were obtained *viz*., F1, F2, F3, F4, F5, F6, F7 and F8, with differences in nature of vegetative growth. Two lines were also developed as protoplast isolates *viz*., POi (protoplast isolate of *P. opuntiae*) and PCi (protoplast isolate of *P. cystidiosus*). Among the fusant lines, F8 took the minimum time for completion of mycelial growth (4.00 days), with white, thick cottony, suppressed mycelium having black colourations and primordia showing stickiness of mycelium on medium followed by F6 (4.33 days), F4 (5.34 days) and F2 (5.44 days). F6 showed white, thick, fluffy and concentric growth with primordial. F4 showed white, thick and fluffy growth, while F3 showed white, dense, suppressed and radiating mycelium in discrete patches. F7 showed slimy, sticky, scattered, crust like, actinomycete growth pattern and couldn't complete their mycelial growth. F3 recorded the maximum hyphal width (3.13 μm) followed by F2 (2.67 μm) and F8 (2.61 μm); and these were significantly greater than parent lines. F2 showed white, thick cottony, suppressed growth and

Table 42. Mycelial characters of parent isolates and protoplast isolates/fusants on PDPA

Sl. No.	Isolates	Nature of mycelial growth	Days for complete growth in petri dish*	Hyphal width (µm)*
1	POi	Pure white, thin cottony, radiating with primordia	5.44^{cde}	1.38^{h}
2	PNCi	Pure white, thick cottony, radiating with primordia	5.01^{def}	2.43^{e}
3	F1	White, light cottony, stranded, radiating from centre	6.33^{c}	1.70^{g}
4	F2	White, thick cottony, suppressed, stickiness of mycelium on medium	5.44^{cde}	2.67^{b}
5	F3	White, dense, suppressed, radiating into discrete patches	22.00^{a}	3.13^{a}
6	F4	White, thick, fluffy	5.34^{cde}	1.93^{f}
7	F5	White, thin, radiating	6.44^{c}	1.20^{i}
8	F6	White, thick, fluffy, concentric with primordia	4.33^{ef}	1.97^{f}
9	F7	Slimy, sticky, scattered, crust like, actinomycete pattern	11.33^{b}	-
10	F8	White, thick cottony, suppressed, with black colourations, primordia and stickiness of mycelium on medium	4.00^{f}	2.61^{c}
11	PO1	Pure white, thick and fluffy with smooth margin	6.33^{c}	1.66^{g}
12	PNC1	Pure white, thick cottony with concentric zonations	6.00^{cd}	2.49^{d}
SE m (±)			1.436	0.182
CD (0.05)			1.132	0.048

POi: *P. opuntiae* protoplast isolate, PNCi: *P. cystidiosus* protoplast isolate, F: Fusant line, *Average of three replications, Means followed by similar superscripts are not significantly different at 5 % level, Treatments without observation not taken for statistical analysis

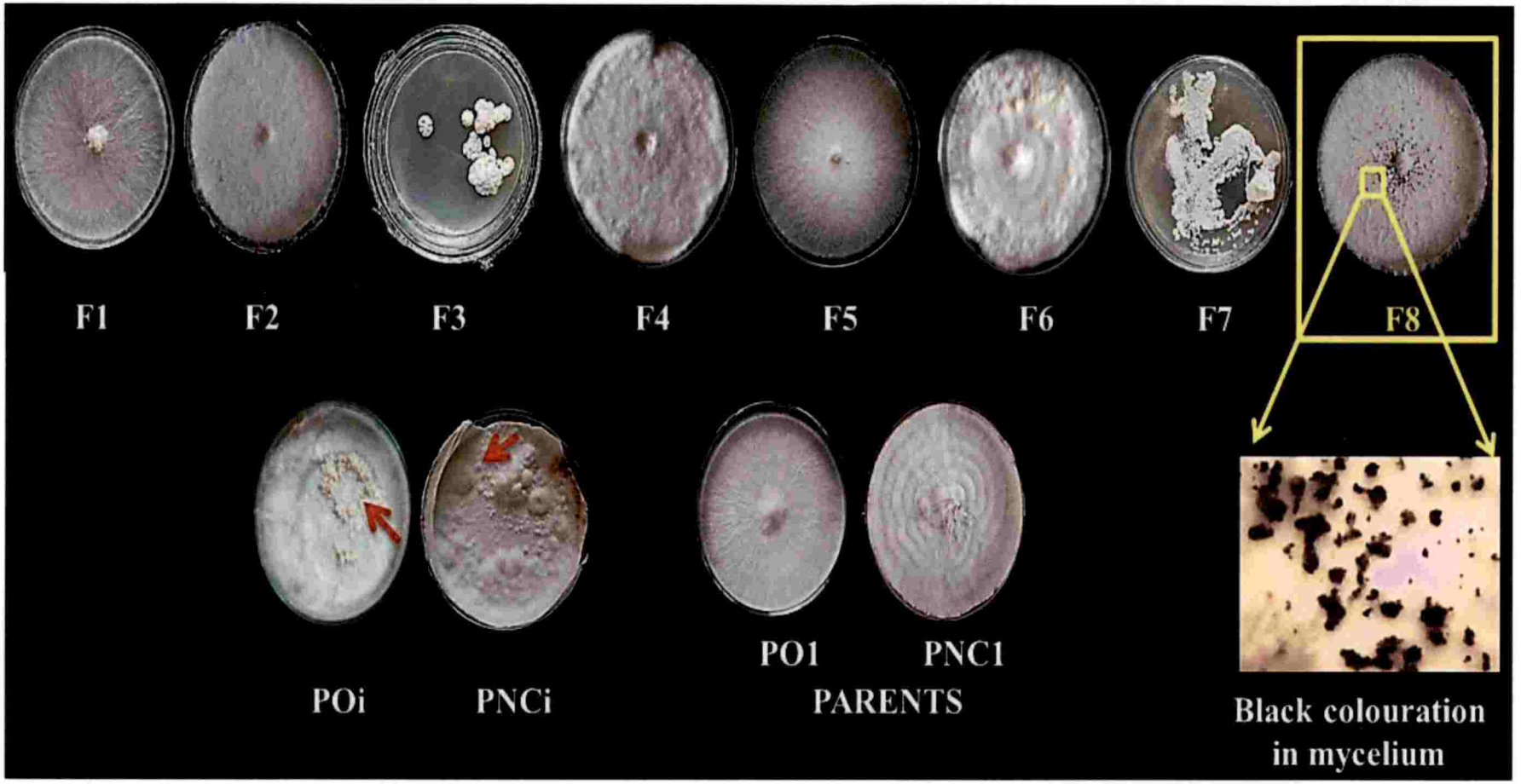


Plate 48. Mycelial characters of parent isolates and protoplast isolates/fusants on PDPA

stickiness on medium. F1 showed white, light cottony, stranded growth radiating from centre whereas, F5 exhibited white, thin, radiating growth. The protoplast isolates *viz.*, POi and PCi had pure white, cottony, radiating, growth with primordia. Clamp connection was found in the parent lines, hybrids and protoplast isolates (Table 42 and Plate 48).

4.18.6.2. Mother spawn production trials with the protoplast isolates and fusants

Among the fusants F6 (8.32 days) took the minimum time for mother spawn production followed by F8 (10.30 days) and F5 (17.4 days). Thicker and fluffy growth was observed in F4, F6, F7, protoplast isolate of *P. opuntiae* and protoplast isolate of *P. cystidiosus* (Table 43 and Plate 49).

4.18.6.3. Cultivation trials with the protoplast isolates, fusants and second generation

A comparison was made between the regenerants and the parent as to primordial initiation time, time for first harvest, number of sporocarps, fresh weight of sporocarps, BE, length of stipe, diameter of stipe, diameter of pileus, incidence of pests and diseases and fruiting temperature. The protoplast isolates of *P. cystidiosus* (non-coremial) and *P. opuntiae* failed to produce fruiting bodies in either paddy straw and sawdust. Maximum fresh weight of sporocarps and BE was recorded with F6 (1677.30 g, 167.80 per cent) followed by *P. cystidiosus* i.e. parent line and F8 (911.25 g, 91.10 per cent). F8 took the minimum time for primordial initiation (16.40 days), followed by F5 (17.20 days), F4 (18.25 days) and F7 (22.20 days). F8 recorded the maximum number of sporocarps (2541.20) followed by F5 (266.30) and F3 (200.00). Fruiting body of F8 was similar to that of *P. opuntiae*, with 5.5 cm x 4.6 cm pileus size and 1.14 stipe length. Fruiting body of F6 was similar to that of *P. cystidiosus* with 14.82 cm x 11.79 cm sized pileus and stipe length of 1.50 cm. Fruiting body of the fusant line F4, showed recombined characteristics of the parental strains, with 9.61 cm x 9.10 cm pileus

Table 43. Comparative performance of parent isolates and protoplast isolates/fusants for mother spawn production in paddy grains

Sl. No.	Isolates	Days for mother spawn production*	Nature of mycelial growth
1	POi	10.62^{i}	++++
2	PNCi	8.62^{k}	++++
3	F1	19.62^{d}	+++
4	F2	12.35^{g}	+++
5	F3	26.35^{a}	++
6	F4	13.35^{f}	++++
7	F5	17.40^{e}	+++
8	F6	8.32^{l}	++++
9	F7	22.07^{c}	++++
10	F8	10.30^{j}	+++
11	*P. opuntiae*	23.68^{b}	+++
12	*P. cystidiosus* (non-coremial)	11.13^{h}	+++
SE m (±)		1.808	
CD (0.05)		0.114	

++++ : Thicker and fluffy growth +++ : Thick growth ++ : Poor growth
*Average of three replications, each replication denotes 5 spawns
Means followed by similar superscripts are not significantly different at 5 % level

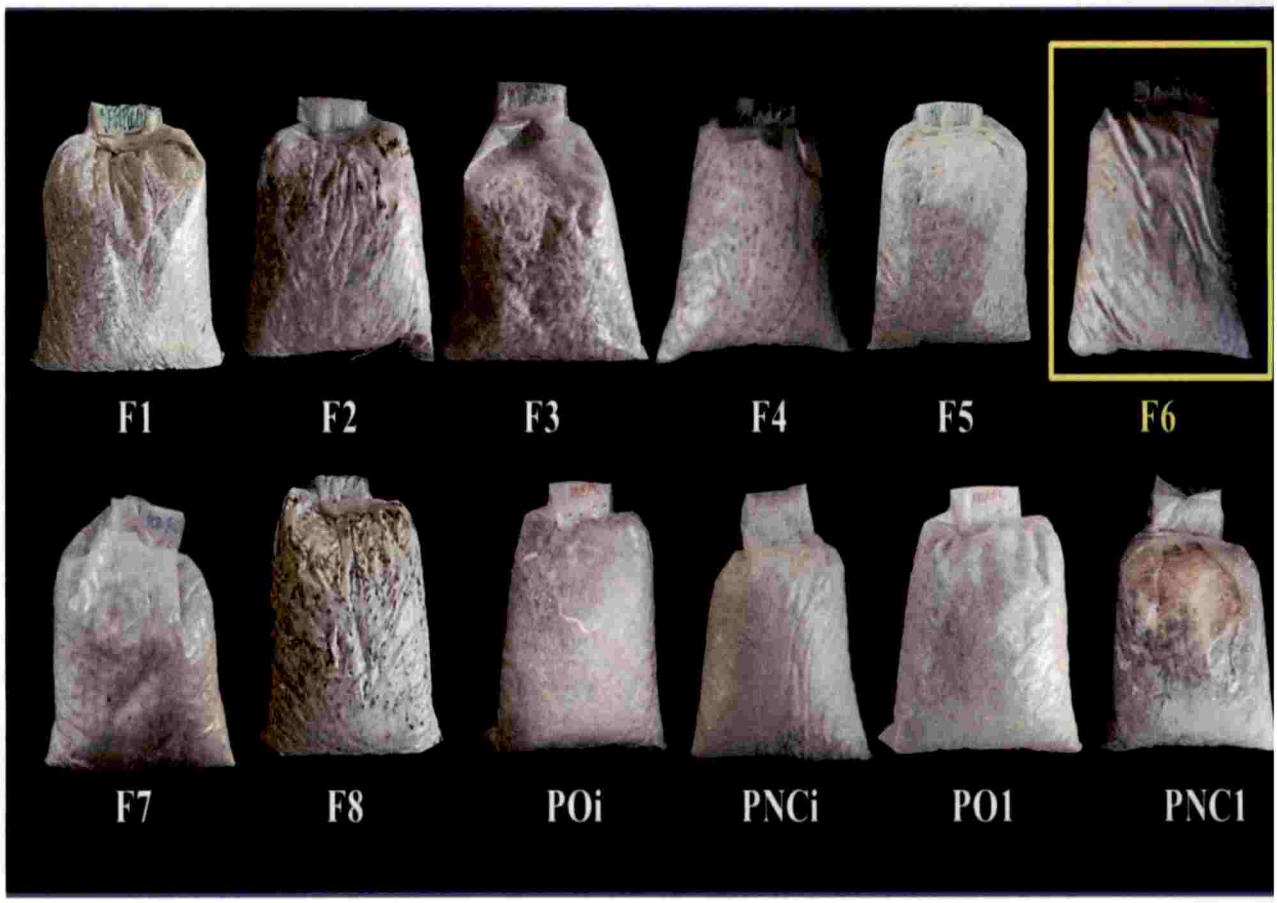


Plate 49. Mother spawns of protoplast fusants/isolates and parents

size and 0.81 cm stipe length (Table 44 and Plate 50). *Trichoderma* spp., staphylinid beeteles and phorid flies (*Megaselia* spp.) were the major pests/fungal contaminants observed in the fusant lines.

The cultivation trials of second generation of the promising fusant lines *viz*., F4, F6 and F8 were carried out. F8 took the minimum time for spawn run and first harvest (16.30 days, 18.05 days), followed by *P. opuntiae* (17.20 days, 21.30 days) and F4 (19.40 days, 22.25 days). Maximum number of sporocarps was recorded with F8 (2787.00), followed by F4 (902.90) and *P. opuntiae* (169.90). Maximum fresh weight and BE was recorded with F6 (1680.35 g, 168.05 per cent) followed by *P. cystidiosus* (1555.30 g, 155.59 per cent). Second generation of F6 and F8 line showed similar morphology to the original fusant lines, with 15.90 cm x 12.88 cm pileus size, 2.70 cm stipe length and 5.50 cm x 4.50 cm pileus size, 1.13 cm stipe length, respectively with comparable BE of 168.05 and 99.95 per cent respectively. However, the fusant line F4 showed segregation in the recombined character and reverted back to the morphology of its parent *P. opuntiae* with 5.90 cm x 5.00 cm pileus size and 1.36 cm stipe length (Table 45 and Plates 50).

Table 44. Comparative performance of parent isolates and protoplast isolates/fusants in rubber wood sawdust

Sl. No.	Isolates	Days for primordial initiation*	Days for first harvest*	Number of sporocarps*	Total yield per bed from three harvests (g)*	BE (%)*	Stipe length (cm)*	Pileus diameter (l cm x b cm)*
1	PNCi	-	-	-	-	-	-	-
2	POi	-	-	-	-	-	-	-
3	F1	25.25^{d}	-	-	-	-	-	-
4	F2	-	-	-	-	-	-	-
5	F3	29.35^{c}	34.25^{c}	200.00^{c}	97.95^{f}	9.70	0.40	3.70 x 2.50
6	F4	18.25^{f}	21.30^{d}	165.55^{f}	485.20^{e}	48.50	0.81	9.61 x 9.10
7	F5	17.20^{g}	20.30^{e}	266.30^{b}	90.25^{g}	9.00	2.53	2.40 x 3.66
8	F6	36.35^{b}	41.10^{b}	180.20^{d}	1677.30^{a}	167.80	1.50	14.82 x 11.79
9	F7	22.20^{e}	-	-	-	-	-	-
10	F8	16.40^{h}	18.20^{g}	2541.20^{a}	911.25^{c}	91.10	1.14	5.50 x 4.60
11	PNC1	51.15^{a}	55.30^{a}	107.95^{g}	1558.30^{b}	155.80	2.89	14.20 x11.20
12	PO1	16.65^{h}	19.40^{f}	170.10^{e}	659.40^{d}	65.90	2.45	8.00 x 6.30
SE m (±)		3.878	5.351	337.544	242.53			
CD (0.05)		0.417	0.452	0.646	0.524			

F: Fusant line, BE: Biological efficiency
* Average of four replications, each replication denotes 5 beds
Means followed by similar superscripts are not significantly different at 5 % level
Treatments without observation not taken for statistical analysis

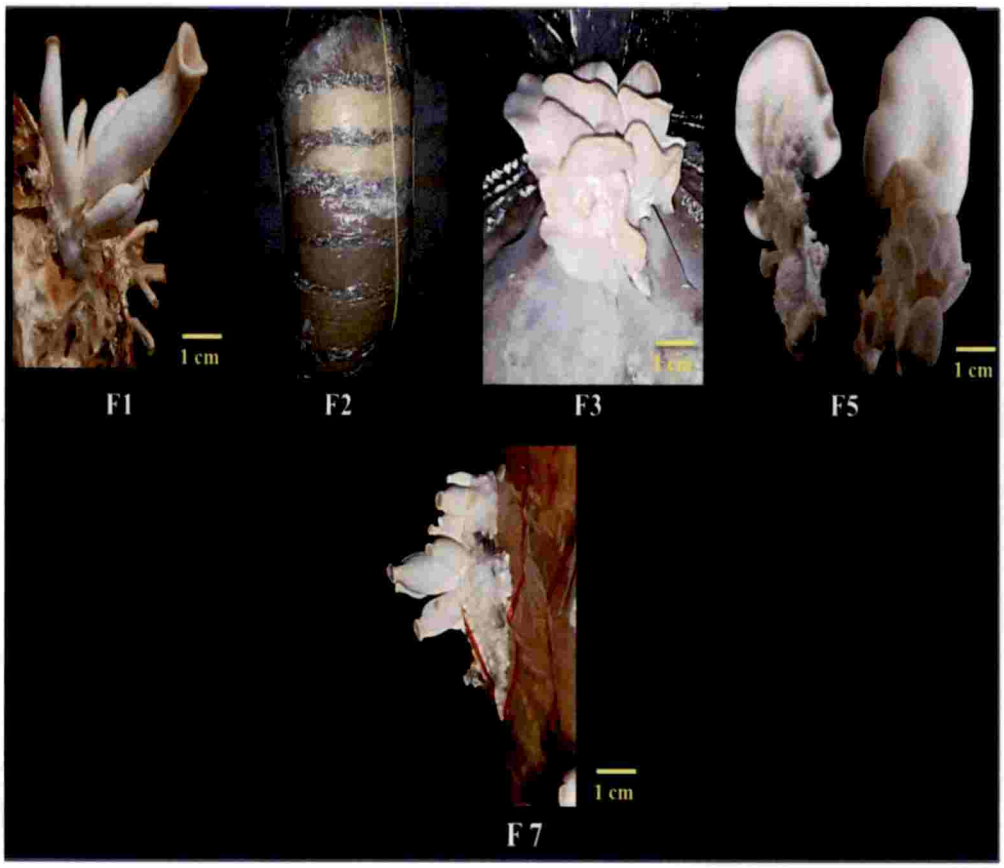
Table 45. Comparative performance of protoplast isolates of parents and fusants (second generation) in rubber wood sawdust

Sl. No.	Second generation	Days for primordial initiation*	Days for first harvest*	Number of sporocarps*	Total yield per bed from three harvests (g)*	BE (%)*	Stipe length (cm)*	Pileus diameter (l cm x b cm)*
1	F4	19.40^{c}	22.25^{c}	902.90^{b}	326.40^{e}	32.41	1.36	5.90 x 5.00
2	F6	38.25^{b}	41.65^{b}	67.75^{e}	1680.35^{a}	168.05	2.70	15.90 x 12.88
3	F8	16.30^{e}	18.05^{e}	2787.00^{a}	1000.25^{c}	99.95	1.13	5.50 x 4.50
4	Parent 1	51.35^{a}	55.50^{a}	103.20^{d}	1555.30^{b}	155.59	2.81	14.10 x 11.00
5	Parent 2	17.20^{d}	21.30^{d}	169.90^{c}	654.35^{d}	65.10	2.46	8.10 x 6.28
SE m (±)		6.988	7.243	518.522	258.366			
CD (0.05)		0.381	0.648	0.969	0.484			

F: fusant line, BE: biological efficiency, Parent 1: *P. cystidiosus* (non-coremial), Parent 2: *P. opuntiae*

* Average of four replications, each replication denotes 5 beds,

Means followed by similar superscripts are not significantly different at 5 % level



Sporocarps of first generation

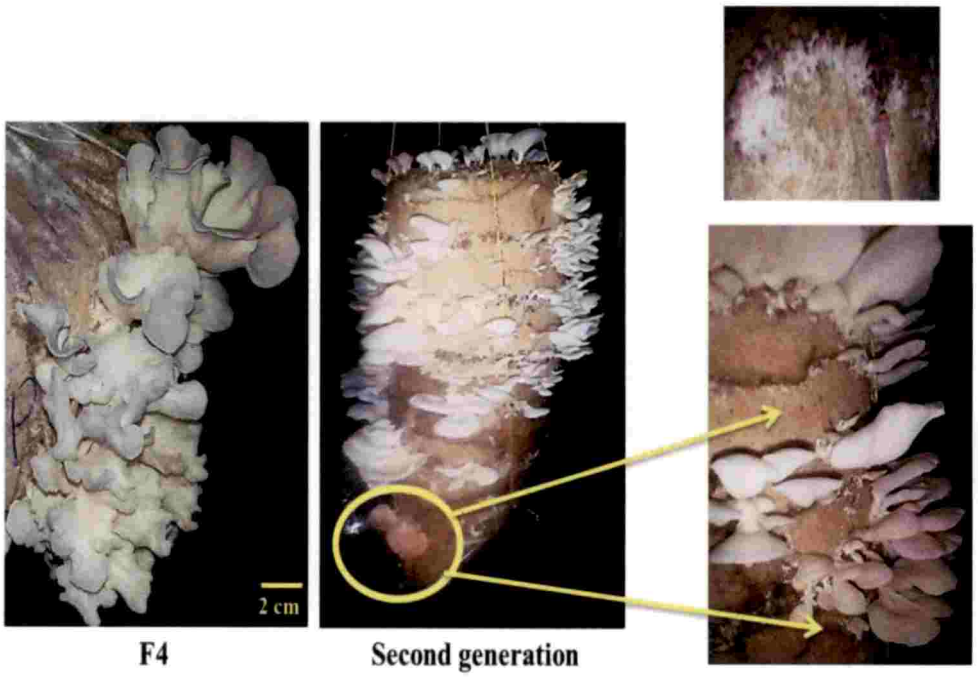
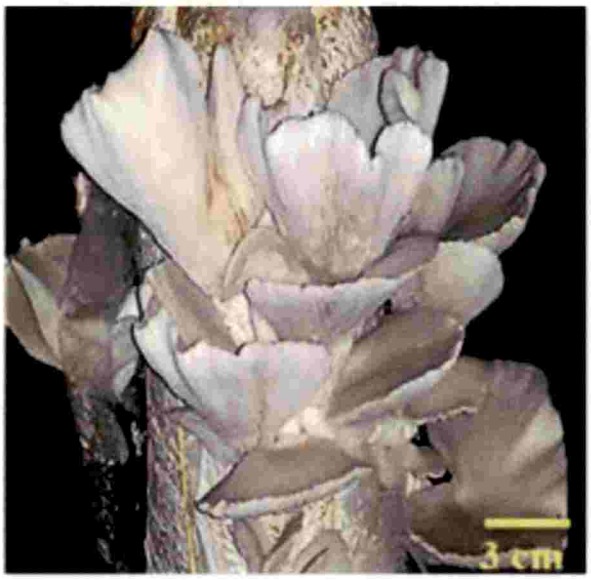
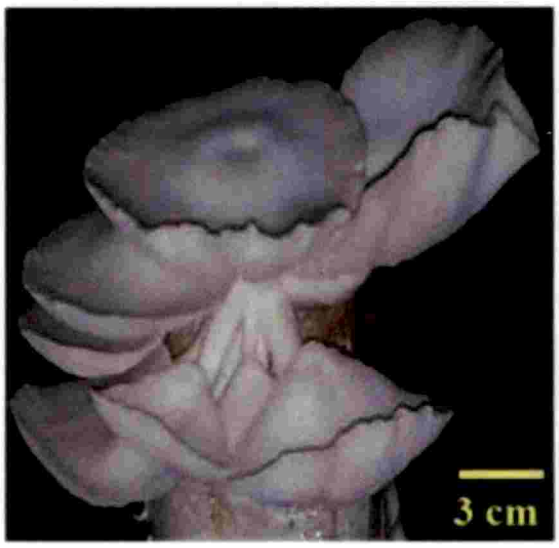


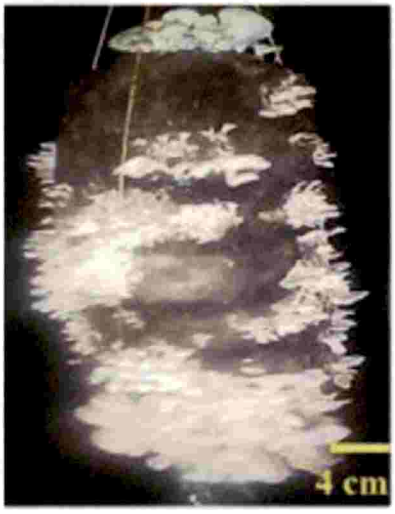
Plate 50. Fruiting body characteristics of fusant lines



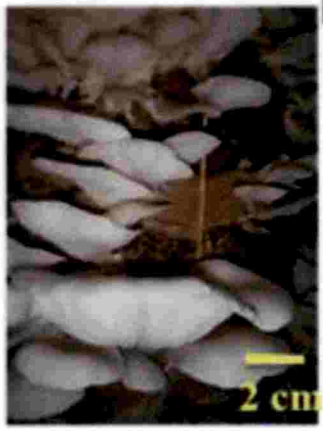
F 6



Second generation



F8



Second generation

Plate 50. Fruiting body characteristics of fusant lines (contd.)

5. DISCUSSION

Mushroom also known as white vegetable or boneless vegetarian meat fall between the best vegetables and animal protein source (Manjunathan and Kaviyarasan, 2011). Among the mushrooms, oyster mushroom (*Pleurotus* spp.), popularly known as 'dhingri' in India, grows naturally in the temperate and tropical forests on dead and decaying wooden logs or on dying trunks of deciduous or coniferous woods. The fruiting bodies of this mushroom are distinctly shell or spatula shaped with different shades of white, cream, grey, yellow, pink or light brown depending upon the species and season. Cultivation of oyster mushrooms was initiated on experimental basis in Germany by Flack during the year 1917 on tree stumps and wood logs. Oyster mushrooms are getting popular in India due to its wider adaptability, easy cultivability, suitability and varied agro-climate in India, abundance of agro wastes, relatively low-cost labour and rich fungal biodiversity. Its tremendous stability of cap and stem, cooking qualities and longer shelf life also enhances its acceptability. Also they have a rich history in traditional Chinese medicine as early as 3,000 years, particularly as a tonic for the immune system, according to acupuncturist Christopher Hobbs, author of "Medicinal Mushrooms".

In Kerala, *Pleurotus florida*, *Pleurotus sajorcaju* and *Pleurotus eous* are the widely cultivated and appreciated species. Lately, two new species of *Pleurotus viz. cystidiosus* and *opuntiae* have been reported from Kerala (Geetha, 2011; 2015). The present study has been undertaken with the objective to standardize the techniques for production of these two oyster mushrooms, *Pleurotus cystidiosus* and *Pleurotus opuntiae* and to study their morphological, physiological and cultural characteristics as well as nutritional and organoleptic qualities and to undertake genetic improvement by protoplast fusion. The post-harvest and anti-cancerous activities of these mushrooms were also undertaken.

5.1. SURVEY

Emergence of mushrooms in the natural ecosystem is a complex phenomenon. Information on mushroom emergence and its season is more useful for mycologists in many ways. It gives an idea about when to look for a particular species of their choice besides providing valuable clues about the overall ecology of the fungi within the natural communities (Pradeep *et al.*, 1998).

Survey for *P. cystidiosus* and *P. opuntiae* identified the coremial isolates of *P. cystidisous* in Vellayani from Ashoka (*Saracca indica)* and Eucalyptus (*Eucalyptus globus*) logs. Non-coremial isolates of *P. cystidiosus* were collected from Chirayinkeezhu, found growing on fallen wood logs of mango (*Mangifera indica*). Another non-coremial isolate was developed from dowel spawns procured from Germany. Isolates of *P. opuntiae* from Vellayani was recorded from mango (*Mangifera indica* L) logs, coconut (*Cocos nucifera* L.) and arecanut (*Areca catechu L.*) logs. All the collected mushrooms were gregarious and lignicolous in nature, except the third isolate of *P. opuntiae* from Vellayani, which was solitary in nature. In line with the findings of present study, Dhancholia (2013) stated that, oyster mushrooms are host dependent and grow on specific host.

P. cystidiosus have been identified from their natural hosts *viz.*, *Schinus terebinthifolius* (Staji *et al.*, 2003), *Ficus bengalensis* (Selvakumar *et al.*, 2008; Vishwakarma *et al.*, 2017), *Mangifera indica* (Atri *et al.*, 2012) and *Spathodea campanulata* (Chaudhary and John, 2017). *P. cystidiosus* subsp. *abalonus* was found growing in caespitose clusters on stump of *Mangifera indica* (Atri *et al.*, 2012), whereas Saha *et al.* (2012); Munoz and Amaya (2013); and Mondal *et al.* (2010) recorded the gregarious nature of *P. opuntiae*. *P. opuntiae* was identified from opuntia, agave, yucca and *Phytolacca* spp. (Saha *et al.,* 2012), arecanut (Geetha, 2015), *Opuntia ficus-indica* (Mondal *et al.*, 2010), *Quercus suber* and *Quercus faginea* (Ali and Stephenson, 2016).

Lignicolous nature of *P. cystidiosus* was recorded by Selvakumar *et al.* (2008); Atri *et al.* (2012); Muruke (2014); Vrinda (2014) and Chaudhary and John (2017). Lignicolous nature of *P. opuntiae* was recorded by Saha *et al.* (2012), Geetha (2015), Ali and Stephenson (2016) and Vishwakarma *et al.* (2017).

5.2. MACROSCOPIC AND MICROSCOPIC STUDIES

Evaluation of morphological and mycelial characters of mushrooms will provide vital information for characterization and selection of strains. Borkar *et al.* (2015) identified the collected mushrooms based on the morphological characters *viz.*, habitat, pileus colour, pileus diameter (cm), pileus shape, stipe diameter (cm), stipe length (cm), spore print and microscopic features.

The morphological studies of *Pleurotus* spp. revealed that coremial and non-coremial isolates of *P. cystidiosus* were light brown to creamish white, oyster shaped, smooth surfaced with fringed margin and pileus size ranging from 18.33 cm x 15.4 cm to 19.70 cm x 16.51 cm and 15 cm x 11.52 cm respectively. A similar observation was made by Stamets (1993) who reported that cap of *Pleurotus abalonus* has a darker colour cap than *P. cystidiosus*. Chaudhary and John (2017) identified sporocarp of *P. cystidiosus,* associated with the bark of tulip tree (*Spathodea campanulata*) on the basis of morphological characters like colour, diameter and shape of pileus; brown to grayish colour, length and diameter of stipe; and attachment of gills. *P. cystidiosus* has pleurotoid, brown to grayish pileus with numerous punctiform squamules, lateral stipe (35-45 to 20-30 mm length and 15 to 28 mm wide) tapered to the base, fleshy when fresh and compact to corky when dry (Madrupji, 2017). Dawidowicz and Siwulski (2017) described the cap of *P. cystidiosus* as convex to hemispherical in shape (2-5 cm wide), cream to off-white in colour, with irregular cap edge and broad, widely spaced, strongly deccurent, irregularly edged gills. Its stem was short, thick, centrally or eccentrically attached.

Sporocarps of *P. opuntiae* were white to creamish white in colour, oyster shaped, smooth surfaced with fringed margin and pileus of size 5.10 cm x 4.02 cm to 9.80 x 5.35 cm. Munoz and Amaya (2013) reported that sporocarps of *P. opuntiae* were initially flat-convex in diameter, then turned to depressed and funnel-shaped with slightly rolled, waved and lobed margin. The collected mushrooms of *Pleurotus* spp. were devoid of volva and annulus, with lateral and fleshy stipe; and free and fleshy gills. This finding is similar to that described by Wasser and Weis (1999).

Spore prints of the *Pleurotus* spp. isolates were white in colour similar to that described by Atri *et al.* (2012); Munoz and Amaya (2013); Pandey and Veena (2012) and Chaudhary and John (2017). Stanley and Nyenke (2011) stated that *Pleurotus* species are characterized by a white spore pint attached to the recurrent gills often with an eccentric stipe.

The studies on the hyphal characters revealed that hyphae of the *Pleurotus* spp. were septate, branched, hyaline, aerial as well as submerged with distinct clamp connection. Similar to the above observation, Miller (1969) described the hyphae of *P. cystidiosus* as hyaline, clamped, thin walled and regularly branched of thickness 1.5-4.2 mm. Milovanovic *et al.* (2014) noted that *Pleurotus ostreatus* has thin-walled, hyaline, branched and anastomosed hyphae with clamp-connections.

In the present study, basidiospores of *Pleurotus* spp. were hyaline, oval, sub cylindrical to cylindrical shape. The basidiospores stained dark blue and red in 1 per cent lactophenol cotton blue and 1 per cent congo red respectively. Miller (1969) described spores of *P. cystidiosus* as hyaline, cylindrical-oblong with thin and smooth wall. Hilber (1982) delineated *P. cystidious* and *P. abalonus* by their spore size. Peterson and Krisai (1999) described basidiospores of *P. opuntiae* as cylindrical to elongate-ellipsoid, smooth, hyaline and thin-walled; with

heterogeneous contents and a size of 9.2-12.0 µm x 4.4-5.6 µm. Atri *et al.* (2012) stained and observed basidiospores of *P. cystidiosus* with 1 per cent congored. Venturella *et al.* (2015) reported that basidiospores of *P. opuntiae* were oval and elongated of size 7.5-10 × 3-5.5 µm.

Sterile or non-spore-producing cells that adorn the gills are called cystidia *viz.*, cheilocystidia and pleurocystidia which help the basidia in their development. They play a key role in maintaining a higher degree of humidity around the gills (boundary layer effect). Their shapes and sizes vary widely between mushroom species and hence used by taxonomists (Kuo, 2006). Cystidial studies revealed the presence of cheilocystidia and pleurocystidia for *P. cystidiosus* (non-coremial), *P. cystidiosus* (coremial) and *P. opuntiae*. These results were in accordance with the studies of Pandey and Veena (2012) and Munoz and Amaya (2013). Saha *et al.* (2012) reported that *P. cystidiosus* has oblong and elliptical basidiospores with clavate pileocystidia and cheilocystidia.

Contrary to the above findings, squamulose pileus surface with pileocystidia and absence of cheilocystidia were recorded for *P. cystidiosus* by Miller (1969). Peterson and Krisai (1999) recorded the absence of pleurocystidia and presence of basidiolar, subclavate, hyaline, thin walled cheilocystidia (28-34 µm x 5.6-6.4 µm) in *P. opuntiae*.

5.3. ISOLATION AND PURE CULTURING

In the current research work, three isolates of *P. opuntiae viz.*, PO1 and PO2, two coremial isolates of *P. cystidiosus viz.*, PC1 and PC2; and two non-coremial isolates of *P. cystidiosus viz.*, PNC1 and PNC2 were obtained through isolation by tissue culturing and pure culturing by hyphal tip method. Isolation by tissue culturing revealed that white hair like strands of mycelial growth started growing from the tissues of PNC1 and PO1 inoculated on PDA medium at two days after inoculation whereas, PC2 took 7 days. Atri *et al.* (2012); Madrupji

(2017) recorded the initiation of hyaline, filamentous, whitish mycelia of *P. cystidiosus* after 4 and 3 days of inoculation respectively followed by the appearance of coremia at 25 ^{0}C.

In the present study, mycelium of PC1 and PC2 isolates showed small protuberances on the entire tissue simultaneously followed by the development of little white stalk with tiny black watery droplets. This agrees with the findings of Bandala and Montoya (1991) who stated that, *P. abalonus* have white mycelia which becomes speckled with black droplets. Stamets (1993) recorded that *P. cystidiosus* is dimorphic, generating asexual structures called coremia (broom-like bundles of spores) *i.e.* stalk-like cells whose tops are fitted with liquid droplets of black spores. The imperfect stage provides the cultivator with the advantage of simultaneous "spore mass" inoculation during inoculation of any substrate with its pure culture spawn. However, coremial liquid do not pose a contamination threat to other cultures in the laboratory until they get dry and harden, during which they become airborne. Also the droplets get streaked across the media, if petri dishes are not handled carefully. Amita and Atri (2017) reported a maximum colony diameter (6.3 cm) of *P. cystidiosus* at the rate of 0.60 cm on an average daily basis in MEA medium after 12 days of inoculation. They observed that, the mycelium formed a thick mat with concentric rings; and in due course coremial droplets were formed on the mycelium mat.

The nature of mycelial growth of *P. opuntiae* isolates was white and thin with or without concentric zonations and radiating margin. DeWit (1959) recorded hyaline, filamentous mycelium with radial and irregular growth; undulated margin; and pinkish zones in *Pleurotus djamor*. Khandakar *et al.* (2008) reported that mycelial extension rate is a reliable technique for the selection of fast growing strains in *Pleurotus* spp. from a given stock of germplasm. The growth rate of hypha could be considered for the characterization of the strain (Liu *et al.*, 2008). The isolates screened for further studies were PC2, PNC1and PO1; and accession numbers *viz.*, DMRO-925 and DMRO-926 were obtained for PO1 and PC2 from Directorate of Mushroom Research (DMR),

ICAR, Solan. Similarly, Barrales and Mata (2016) selected two strains of *P. opuntiae viz.*, IE 837 and IE 863 from nine strains obtained from maguey plants (*Agave salmiana*) in Mexico. Dawidowicz and Siwulski (2017) selected strain B1 of *P. cystidiosus* based on faster growth of mycelium and hyphal quality when incubated at 15 to 30 ^{0}C.

5.4. MOLECULAR CHARACTERIZATION OF *PLEUROTUS* SPP.

The use of molecular tools is essential to ensure the correct identity of inoculum used. Molecular tools provide more accurate methods for identification than the few characters afforded by the traditional morphological features. Within the rDNA locus, the ITS (Internal transcribed spacer) region has been particularly useful for analysis of closely related species in many genera of cultivated mushrooms (Dung *et al.*, 2012).

In the present study, PC2, PNC1, PO1, *P. florida* and *P. eous* were molecularly characterised by ITS sequencing and accession numbers *viz.*, KY214254, KY887023, KY214255, KY214256 and KY214257 respectively were retrieved from NCBI GenBank. Similarly, Atri *et al.* (2012) carried out ITS sequencing studies with *P. cystidiosus* which showed highest identity with *P. cystidiosus* strain ACCC 51280. Dung *et al.* (2012) morphologically and molecularly characterised two strains of *Pleurotus floridanus* and one strain of *P. cystidiosus*. Shnyreva *et al.* (2017) characterised *P. cystidiosus* strain AG 55/466 wild-type isolate and accession number *viz.*, FJ608592 was retrieved from GenBank.

Comparative nucleotide sequence alignment revealed that, PO1 showed 90 per cent identity with *P. djamor*. *P. djamor* is an inter-sterile group commonly found in Mexico and is formed of three different phenotypic carpophores *viz.*, white, grey and pink; denominated as *djamor*, *opuntiae* (Petersen and Ridley, 1996) and *salmoneostramineus* (Toro, 2002) respectively. Contradictory to the above findings, Ro *et al.* (2007) reported that sequence-based analysis is not

applicable for the verification of closely related mushroom strains *viz.*, *P. ostreatus* and *P. ferulae*.

Phylogenetic analysis revealed that, PC2, PNC1 and PO1 grouped under a larger cluster and was distantly related to *P. florida* and *P. eous* (Fig 1). Similarly, Stamets (1993) reported that *P. cystidiosus* shares greatest similarity from a cultural viewpoint with *P. abalonus*. However, Hilber (1982) stated that a combination of features delimited *P. abalonus* from *P cystidiosus*. Chaudhary and John (2017) recorded the similarity of *P. cystidiosus* with *Pleurotus cystidiosus* strain P24 based on nucleotide homology and phylogenetic analysis followed by the inference of evolutionary history using Neighbour-Joining method.

5.5. PHYSIOLOGICAL STUDIES OF PLEUROTUS SPP.

Optimum growth rate of mycelium is important for successful production of mushrooms and several secondary metabolites. The growth of mycelium depends on several factors such as growth media, pH, temperature, light/dark conditions and nutrient elements. Hence, the fast growing isolates of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* were subjected to physiological studies.

5.5.1. Growth of *Pleurotus* spp. in different media

Growth medium is the most important factor because it supplies necessary nutrients for the growth of mycelium. PDPA was identified as the best medium for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* (Fig 2). The mycelium of *P. cystidiosus* produced black coremial asexual structures and took longer time to complete the growth in all media. These findings were similar to the results reported by Nasim *et al.* (2001). Yasotha (2008) noted that *P. cystidiosus* produced black heads of coremia on MEA, SSM and RC whereas, pinkish pigment was observed in the mycelia of *P. djamor*. The difference in mycelial growth on different media may occur due to availability of different carbon sources and other required nutrients as stated by Madrupji (2017).

Suharban and Nair (1994) reported that oat meal and potato dextrose agar supported superior mycelial growth of *Pleurotus* spp. Pandey *et al.* (2016) identified wheat extract agar (WEA) as the best media for *P. cystidiosus* followed by rice bran agar (RBA) and MEA with mycelial dry weights of 4.91, 4.39 and 3.94 mg respectively. Zagrean *et al.* (2016) reported that, *Pleurotus eryngii* gave the highest mycelial growth rate on MEA followed by PDA and WEA.

Contrary to the above findings, Kashyap *et al.* (2015) recorded OMA as the best medium for the growth of *P. florida* compared to MEA, OMA, WEA and RBA. Madrupji (2017) recorded that, after 14 days of inoculation, the mycelial growth of *P. cystidiosus* was significantly more on OMA (42.00 mm), followed by PDA (40.00 mm) and Richards' agar (33.00 mm).

The above media studies recorded the initiation of sporocarps in *P. opuntiae* on PDPA at 8 days after incubation. Similarly, Srivastava (2000) observed initiation of sporophores after 60 days on PDPA medium. Stamets (1993) listed the following species *viz., Pleurotus citrinopileatus*, *djamor*, *ostreatus*, *eousmus*, *pulmonarius*, *Hypsizygus ulmarius*, *Ganoderma lucidum* and *Agrocybe aegerita* which could produce primordia on 2 per cent enriched MEA, supplemented with yeast 0.2 per cent and gentamycin sulphate 0.005 per cent. This may be because mycelial networks when disturbed at primordia formation resulted in vigourous growth and subsequent number of primordia.

5.5.2. Growth of *Pleurotus* spp. at different temperature

Temperature has a profound effect both on growth and reproduction of fungi (Kaul *et al.*, 1978); and 30, 25-30 and 25 ^{0}C were identified as the best temperatures for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae*, respectively (Fig 3). Atri *et al.* (2012) recorded the optimum temperature for mycelium growth of *P. cystidiosus* at 25 ±1 ^{0}C and no coremia formation was recorded below 15 ^{0}C. Dung *et al.* (2012) recorded superior mycelial growth of *P. cystidiosus* and *P. floridanus* at 27 ^{0}C. *P. sapidus* (FW-133) and *P. cystidiosus* recorded the optimum mycelial growth at 35 ^{0}C (Sardar *et al.*,

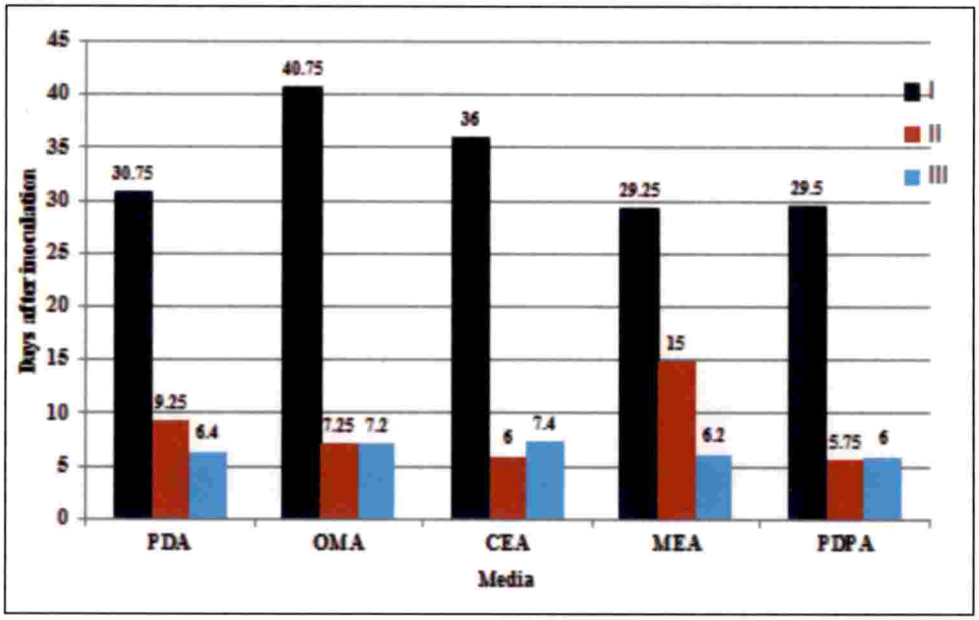


Fig 2. Mycelial growth of *Pleurotus* spp. on different media

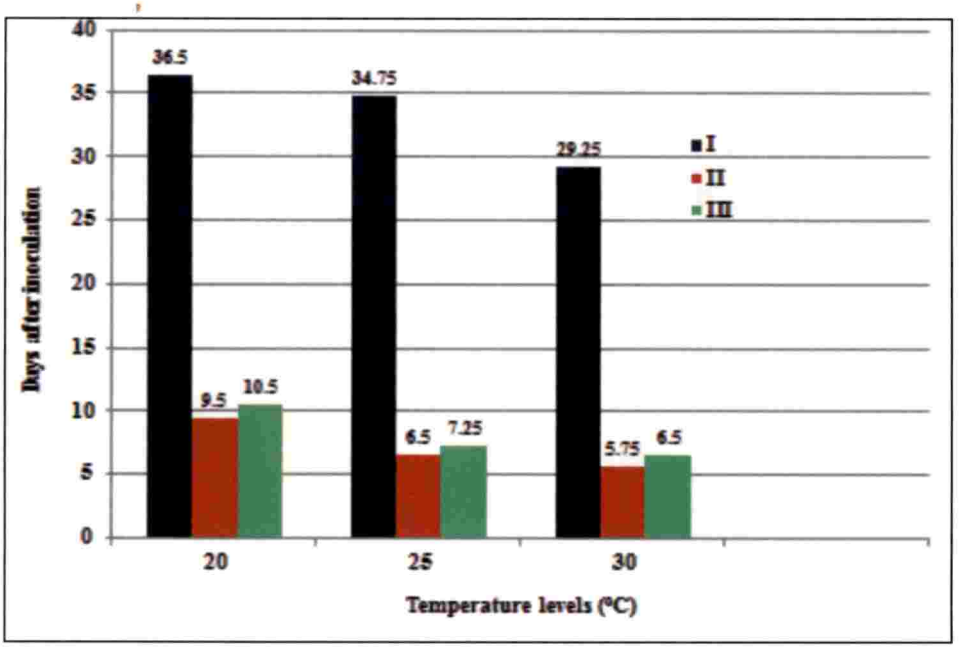


Fig 3. Influence of temperature on mycelial growth of *Pleurotus* spp.

I: *P. cystidiosus* (coremial), II: *P. cystidiosus* (non-coremial), III: *P. opuntiae*

2016) and 28 ^{0}C (Hoa and Wang, 2015). Pandey *et al.* (2016) reported the optimum temperature for the mycelial growth of *P. cystidiosus* as 18-33 ^{0}C. Amita and Atri (2017) recorded the maximum mycelial growth of *P. cystidiosus* in MEA (6.3 cm) and yeast glucose medium (8.3 mg ml^{-1}), giving dense and thick mycelium at 30 ^{0}C, whereas no mycelial growth was noted at 15 ^{0}C and 40 ^{0}C. Dawidowicz and Siwulski (2017) recorded the optimum temperature of incubation for *P. cystidiosus* as 25 ^{0}C whereas mycelial growth was significantly lower at 30 to 20 ^{0}C. Madrupji (2017) reported that the mycelial growth of *P. cystidiosus* was significantly more at 30 ^{0}C (56.00 mm) followed by 25 ^{0}C (29.00 mm), 20 ^{0}C (15.75 mm) and 35 ^{0}C (10.00 mm).

Kumla *et al.* (2013) observed that *Pleurotus* spp. was able to grow at a temperature ranging from 15-35^{0}C with an optimal growth temperature of 25 ^{0}C. Yadav and Chandra (2014) recorded the optimum mycelial growth of *Pleurotus* sp. at 25^{0}C followed by 30, 20 and 15 ^{0}C.

5.5.3. Growth of *Pleurotus* spp. in different pH

pH is one of the most important environmental factor that alters the growth and extension of fungal mycelia (Akinyele and Adetuyi, 2005). Evaluation of different pH levels revealed that pH 8 recorded the highest mycelial growth for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* (Fig 4). The results is consistent with the findings of Pandey *et al.* (2016) who recorded pH 7.5 as optimum for the maximum dry weight of mycelium of *P. cystidiosus* (381.3 mg). Yadav and Chandra (2014) identified yeast peptone dextrose agar of pH 8 at 25 ^{0}C as the best which recorded the fastest mycelial growth of 5 different strains of *Pleurotus sp.* This may be because biomass decreased in a highly acidic or alkaline environment due to high mycelial toxicity (Rawte and Diwan, 2011). Contrary to the above findings, Hasan *et al.* (2015) stated that most of the species of *Pleurotus* require an optimum pH of 5.6 to 6 for best mycelial growth. Zagrean *et al.* (2016) reported that *P. eryngii* preferred media with a weak acid to the neutral reaction with the optimal pH of 6.0. Amita and Atri (2017) recorded that

MEA and yeast glucose medium with pH 6.5 recorded the maximum growth rate of *P. cystidiosus i.e.* 0.47 cm day^{-1} and 12.9 mg ml^{-1} respectively whereas, minimum growth was recorded with pH 4.0 (0.18 cm day^{-1} and 3.1mg ml^{-1} respectively).

5.5.4. Growth of *Pleurotus* spp. in light and dark conditions

Light is reported to act as a signal for triggering various biophysical and biochemical processes leading to morphological reactions. It exerts a significant impact on the growth and development process of carpophores of *Pleurotus* spp., along with other external features (Trukhonovets, 1991). The result showed that darkness is more effective when compared to artificial light in terms of mycelial growth for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* (Fig 5). Similar observations were reported by Vetayasuporn (2016) who observed that *Pleurotus* genera preferred total darkness followed by natural diffused day light.

Ibekwe *et al.* (2008) revealed that the absence of light gave the greatest mycelial growth in *Pleurotus* spp. followed by alternation of light and darkness however, continuous light decreased growth rate. Rout *et al.* (2015) reported that 200 lux light induced the highest linear mycelial growth (63.92-78.89mm) for *Pleurotus* species *viz., eous*, *florida*, *sajor-caju*, *citronopileatus*, *fossulatus*, *flabellatus*, *platypus*, *ostreatus* and *H. ulmarius.* Amita and Atri (2017) identified that dark condition supported the best mycelial growth of *P. cystidiosus* in MEA and yeast glucose medium (YGM) (6.3 cm and 7.63 mg ml^{-1} respectively) compared to light condition (5.06 cm and 6.46 mg ml^{-1}). On contrary, Bumanlag *et al.* (2018) noted that *P. djamor* recorded no difference in mycelial growth in light or dark condition.

It was found that more number of coremia was formed by *P. cystidiosus* (coremial) at 1400 Lux illumination compared to complete darkness. Similiarly, Atri *et al.* (2012) recorded that formation of coremia in *P. cystidiosus* was induced

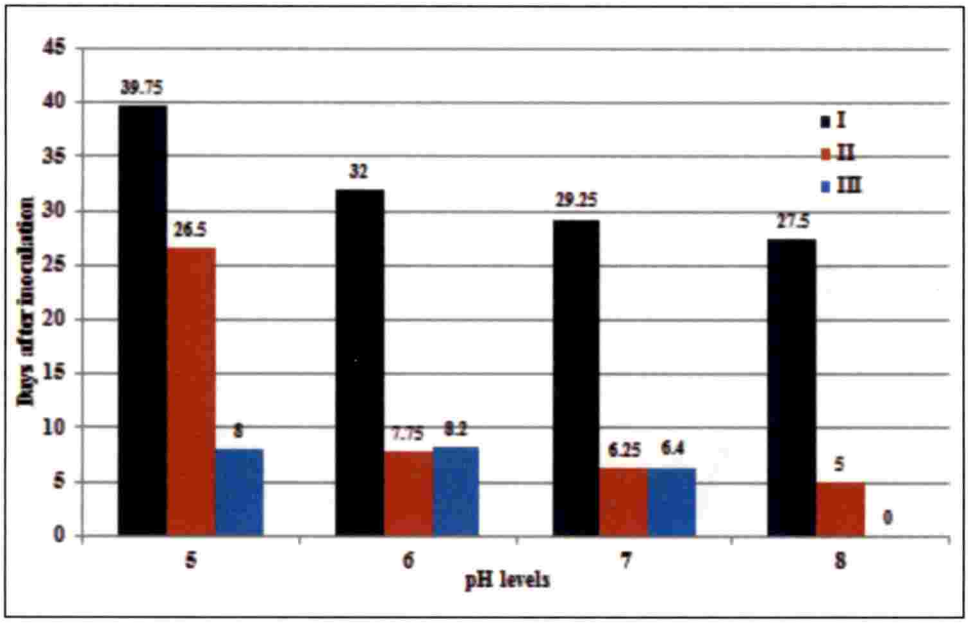


Fig 4. Influence of pH on mycelial growth of *Pleurotus* spp.

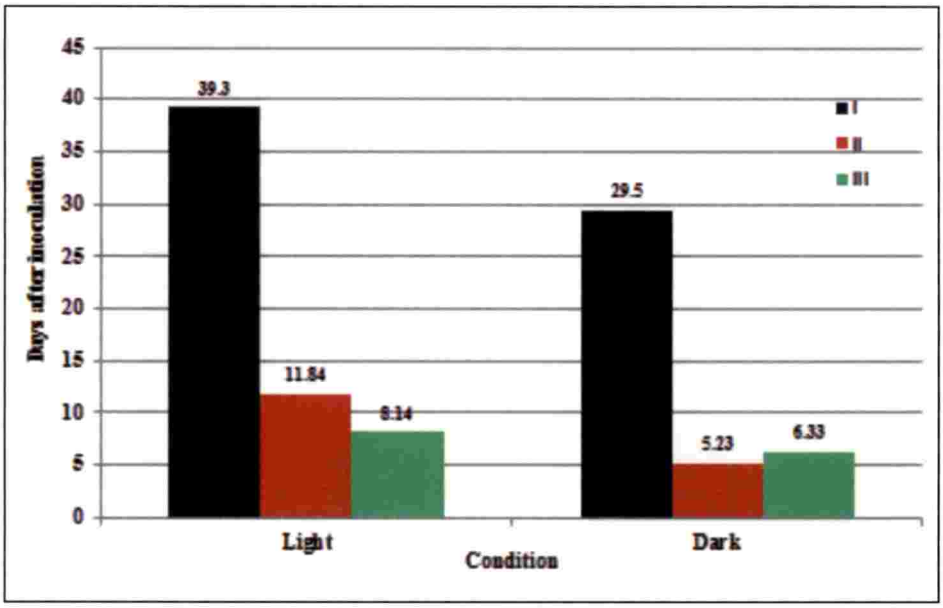


Fig 5. Influence of light and dark conditions on mycelial growth of *Pleurotus* spp.

I: *P. cystidiosus* (coremial), II: *P. cystidiosus* (non-coremial), III: *P. opuntiae*

by light and maximum amount of coremioliquid production was observed under 400 lux continuous illuminations at 25±1 ^{0}C.

5.5.5. Amendment studies of *Pleurotus* spp.

Microorganisms require trace elements and nutrients for their growth rate, metabolic processes and sustenance in other to overcome severe physiological consequences (Seth and Taga, 2014). Hoa and Wang (2015) stated the necessity to furnish those compounds in the media which are required for its growth and other life processes in order to culture oyster mushrooms in laboratory.

The amendment studies were done with PDPA and one per cent yeast was identified as the best amendment for the *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* based on the minimum time taken for complete coverage of petri dish and thickness of mycelial growth. The positive effect of yeast extract in the mushroom production was also reported in *Grifola frondosa* and *Auricularia polytricha* (Lee *et al.*, 2004) and *P. sajor-caju* (Confortin *et al.*, 2008). Uddin *et al.* (2011) recorded that *P. florida*, *Calocybe indica* and *Agaricus biporus* recorded the maximum mycelial proliferation in yeast potato dextrose agar medium. Owaid *et al.* (2014) recorded maximum mycelial growth rate and initiation of primordia of *Pleurotus salmoneostramineus* (12.90 mm day^{-1}) in PDA supplemented with 1 g L^{-1} of extract of *Saccharomyces cerevisiae*. Hong (2016) recommended that, 'YP600' a product prepared from deep-seated fermentation broth of baker's yeast through vacuum concentration and spray drying can replace the constituents of traditional medium *viz.*, bran, sugar, corn meal, soybean meal and other nutrients.

Pokhrel and Ohga (2007) listed out the various organic nitrogen sources suitable for growth of mushrooms *viz.*, meat peptone, polypeptone, yeast extract and soybean concentrates. Yadav and Chandra (2014) and Almeida *et al.* (2015) recorded that yeast extract provided higher mycelial biomass production in *P. ostreatus* with optimal point at 2.96 g of nitrogen L $^{-1}$; and its appropriateness can

be associated to its microbial origin and distribution of aminoacids closer to fungal composition. Also yeast is rich in proteins, peptides, amino acids, nucleotides, vitamins and trace elements. Contrary to the above findings, Ahmad *et al.* (2015) recorded the highest mycelial growth rate (0.24 cm) of *P. djamor* in PDA media (0.24 cm) and the lowest growth (0.11 cm) in yeast media.

In the present study, minimum or no mycelial growth was recorded when iron was used as amendment. This may be because ferrous iron, in particular, is a strong pro-oxidant owing to the electron transferring capacity of iron as noted by Gutteridge and Halliwell (1989). Oghenekaro *et al.* (2008) stated that when iron is present in high amounts, it can release free radicals through Fenton's reaction. These radicals can cause enzyme inhibition, dislocation or substitution of essential ions and membrane rupture which affects the fungal metabolism. In line with the present study, Ogidi *et al.* (2016) reported that mycelial growth rate and biomass produced by *Pleurotus* species *viz., ostreatus*, *cornucopiae*, *djamor*, *pulmonarius* and *djamor* v. *roseus* decreased as iron concentration increased from 0 to 100 mg L^{-1}. Yokota *et al.* (2016) recorded a decrease in BE of *P. ostreatus* when substrates were supplemented with more than 100 mg kg^{-1} of iron.

5.6. SUBMERGED CULTURING OF *PLEUROTUS* SPP.

Dulay *et al.* (2016) revealed that submerged cultivation of mycelial biomass for bioactive metabolites and biomass is more advantageous since mushroom can produce high BE in a short incubation period within reduced space, with less chance of contamination and better control of physicochemical parameters.

The submerged culture production of isolates revealed that PDPB recorded the highest fresh weight and biomass for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* (Fig 6). The characteristics of the mycelium produced by submerged fermentation are influenced by culture conditions *viz.*, composition and initial pH of the fermentation medium; age and size of inoculum;

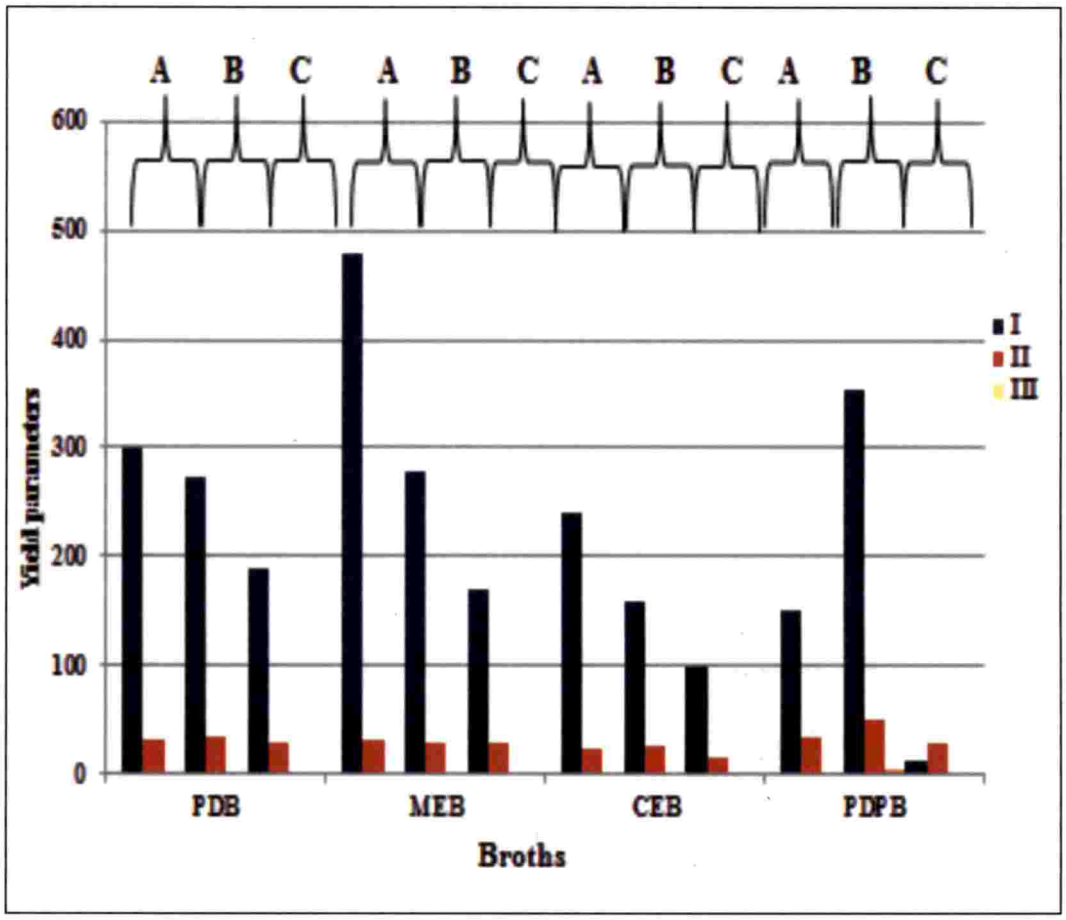


Fig 6. Evaluation of submerged culture production of *Pleurotus* spp.

I: No. of pellets/flask, II: Fresh weight, g, III: Biomass (dry weight, g 100 ml^{-1})

A: *P. cystidiosus* (coremial), B: *P. cystidiosus* (non-coremial), C: *P. opuntiae*

aeration rate and agitation speed (Znidarsic and Pavko, 2001 and Kim *et al.*, 2006). The production of mycelial biomass and valuable polysaccharides of *Pleurotus* spp. varies with the species, their growth parameters, growth timing and nutritional requirements (Gregori *et al.*, 2007). Horincar *et al.* (2014) recorded the optimum dry biomass yield of *P. ostreatus* (7.58 g L^{-1}) in dextrose (40 g L^{-1}), peptone and yeast extract (1:1) medium with 0.2 per cent inoculum concentration at 26 ^{0}C and 150 rpm. The optimal submerged culture condition for *P. florida* was recorded with glucose (20 g L^{-1}), yeast extract (4 g L^{-1}), potassium dihydrogen phosphate (0.46 g L^{-1}), dipotassium hydrogen phosphate (1 g L^{-1}) and magnesium sulphate (0.5 g L^{-1}), at 28 ^{0}C and pH 8 (Masra, 2014). Kirsch *et al.* (2016) recorded the highest mycelial biomass production (9.81 g L^{-1}) of *Pleurotus albidus* in the medium formulated with saccharose (30.0 g L^{-1}) and yeast extract (2.5 g L^{-1}) at pH 7.0 and 180 rpm speed of agitation. Amita and Atri (2017) recorded that yeast glucose liquid medium supported the maximum yield for *P. cystidiosus* (7.9 mg ml^{-1}) followed by PDB (6.9 mg ml^{-1}) whereas, minimum growth was recorded in Asthana-Hawker medium (0.08 mg ml^{-1}) and absence of growth in Will Mineral Salt medium.

In the present study, mycelial biomass developed in two forms *viz.*, filamentous and pellet. Mycelial pellets were small, medium to big sized, light creamy to white coloured and were formed by the development of mycelia into spherical aggregates *i.e.* branched and partially intertwined network of hyphae as described by Park *et al.* (2002); Kim *et al.* (2010). The filamentous form consisted of viscous, homogenously spread hyphae in the culture medium. The mycelial biomass thus produced by submerged fermentation can be used as an inoculum source for mushroom production in semi-solid fermentations and for extraction of antimicrobial compounds, polysaccharides and antioxidants. During the fermentation process, the biomass of *Pleurotus* spp. was observed to adhere strongly to the inside walls of the Erlenmeyer flasks as described by Kirsch *et al.* (2016) for *P. albidus*. This phenomenon was also observed in submerged

fermentation of *G. lucidum* due to the production of polysaccharides (Wagner *et al.*, 2004). Kirsch *et al.* (2016) recorded a higher mycelial biomass production of *P. albidus* when the pellets were smaller in size.

5.7. COREMIAL CHARACTERS OF *P. CYSTIDIOSUS*

In the present study, *P. cystidiosus* (coremial) was characterised by the specialised feature named coremia representing its anamorphic stage, *Antromycopsis macrocarpa*. Microscopical observation of the aerial hyphae showed stalk-like cells whose tops were fitted with black liquid droplets. Black coremial liquid comprised of elliptical and round spores of sizes 16.31 µm x 7.48 µm and 8.06 µm x 8.49 µm respectively. Similar to the current observation Miller (1969) described the coremia of *P. cystidiosus* as black in colour, caespitose composed of a globose head (0.4-0.9 mm diameter) and a stem (0.6-1.25 mm long and 0.1-0.2 mm diameter); formed of thin-walled clamped hyphae, which slowly disarticulated at the ends into fuliginous and cylindrical conidia (5.2-6.2 mm and 14.5-17 mm). Guzman *et al.* (1991) described coremia as bundles of parallel growing erect hyphae (prosenchyma) which together form a stalk and release large numbers of chains of brown coloured arthroconidia (4-7 mm size) from the upper hyphal tips. The released conidia forms a mucous liquid at the loosened apex resulting in shiny black heads in the culture. Truong *et al.* (2006) recorded that coremia of *P. cystidiosus* subsp. *abalonus* produced approximately 4,00,000 arthroconidia per coremium per day with the remnant of a clamp connection and constant productivity over a 2-week period. He also recorded a continuous cell extension and division in the coremium stipe which supplied cells for arthroconidiation at the coremium apex surrounded by a liquid droplet (coremioliquid).

5.7.1. Isolation and characterization of melanin from *P. cystidiosus*

Melanins are dark (usually black), complex, poorly characterized pigments, synthesized enzymatically or auto-oxidatively from a variety of cyclic, heterocyclic, phenolic or other resonance stabilized precursor molecules (Vallimayil and Eyini, 2013). Melanins are difficult to characterize because of

their intractable chemical properties and heterogeneity in their structural features due to the presence of many complex conjugated structures in the melanin molecule (Cockell and Knowland, 1999). In the present study melanin pigment from coremial liquid of *P. cystidiosus* was extracted and characterised by bio-chemical tests and UV spectra. UV spectrum showed a characteristic absorption peak around 250 nm which was typical of the absorption profile of melanin. Also a linear decrease in the absorption with increasing wavelength was observed as described by Arun *et al.* (2015) (Fig.). Similarly Suryanarayanan *et al.* (2004) and Selvakumar *et al.* (2015) carried out biochemical characterisation of melanin extracted from *P. cystidiosus* and recorded absorption peak in the UV regions ranging from 250 to 300 nm.

5.7.2 Antioxidant and antiproliferative activity of melanin from *P. cystidiosus*

Melanin is one of the few known stable free radicals which can act as a sponge for other free radicals that result from environmental stress. Its antioxidant potential is attributed to the complex molecular structure (Bell and Wheeler, 1986). Free radical scavenging is a mechanism which inhibits lipid oxidation and is used to estimate antioxidant activity (Umamaheshwari and Chatterjee 2008). DPPH is a stable free radical with good absorption at 517 nm and is used to study the radical scavenging activity of extracts. When antioxidants donate proton to these radicals then absorption of samples decreases and radical scavenging activity is measured by decrease in absorption (Srivastava *et al.*, 2006).

In the present study, extracted melanin showed efficient free radical scavenging activity of DPPH radical. Reduced absorbance at 516 nm with different doses of melanin was observed. This may be due to the reduction of the DPPH molecules and electron transfer from melanin suspension (Suryanarayanan *et al.*, 2004). The results were similar to those described by Selvakumar *et al.* (2008) in *P. cystidiosus*, Arun *et al.* (2015) in *Schizophyllum commune* and Zou *et al.* (2015) in *Auricularia auriculae*.

5.8. EVALUATION OF DIFFERENT SUBSTRATES FOR SPAWN PRODUCTION OF PLEUROTUS SPP.

The choice of substrate for spawn production is made after considering several factors such as its prevailing price, easy supply and particle size. Spawn production and amendment studies revealed that, yeast 1 per cent was the best amendment in paddy grain, sorghum and rubber wood saw dust for the three isolates. This may be because yeast extract supplies vitamins, proteins, carbohydrates and micronutrients (Zimbro *et al.*, 2009).

Best substrate and amendment for *P. cystidiosus* coremial and *P. opuntiae* was identified as sorghum with yeast one per cent whereas, paddy grains with yeast one per cent was the best for *P. cystidiosus* (non-coremial). However spawns prepared from rubber wood sawdust amended with yeast 1 per cent recorded the maximum shelf life for *P. cystidiosus* (non-coremial), *P. cystidiosus* (coremial) and *P. opuntiae*. In line with the present work, Asghar *et al.* (2007) identified sorghum as the best spawn substrate for *P. sajor-caju*, which took 7 days for spawn run. Narh *et al.* (2011) showed that the combination of sorghum and millet grains in 3:1 (w/w) ratio showed the fastest mycelial growth of *P. ostreatus* (16 days) followed by sorghum (18 days). Sahu (2012) reported that sorghum (7.33 days), paddy grain (8.66 days) and maize grains (9 days) were the best substrates for spawn development of *P. eous.* Savaliya (2014) reported the highest reduction in weight of spawn after mycelial colonization in sorghum grain followed by maize, wheat and bajra. Barrales and Mata (2016) prepared spawns of *P. opuntiae* in sorghum seeds and incubated at 25 ^{0}C in dark for 21 days. Thongklang *et al.* (2015) identified sorghum as the best spawn substrate for *P. ostreatus*. Madrupji (2017) recorded that *P. cystidiosus* took 3.25, 3.75, 4.00 6.00 and 6.75 days for initiation of mycelium run on maize, bajra, finger millet, wheat and sorghum grain spawn respectively. They also identified maximum preferability for spawns prepared from sorghum and wheat in *P. cystidiosus*, as it showed the maximum reduction in weight due to loss of moisture from grain spawn and nutrients uptake.

Minimum spawn run and shelf life was recorded with sorghum grains followed by paddy grains and rubber wood sawdust for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae*. This may be because, spawn run rate of smaller grains was higher than the larger grains whereas, larger grains have a greater food reserve (Elliot, 1985) and can sustain the mycelium for longer periods of time during stress with maximum shelf life (Fritsche, 1988). Mamiro and Royse (2008) noted that small size of millet provides a large surface area and greater number of inoculation points for the fungal mycelium per kg than larger paddy grains and sawdust. Jongman *et al.* (2013) identified sorghum grains as the best spawn substrate in terms of mycelium growth vigour rating, (5.25) colonization time (7 days), spawn running time (12 days) and BE (75.40 per cent) for hybrid between *P. ostreatus* and *P. florida*, followed by wheat (2.45, 17 days, 16 days and 53.55 per cent) and barley grains (3.46, 14 days, 13 days and 55.24 per cent).

Contaminants are one of the major problems in mushroom spawn production. Maximum contamination was recorded with spawns prepared from paddy grains, sorghum and rubber wood sawdust for *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae*. Major contaminants observed with paddy grains were *Aspergillus* sp., *Trichoderma* sp., *Bacillus* sp., *Rhizopus* sp., *Mucor* sp. and *Penicillium* sp. Paddy grains is very nutritious for fungi and form kernels easily which can be easily distributed to the substrate. However, chances of contaminations are much higher compared to sawdust or wooden stick spawns because it provides optimal substrate for other micro-organisms (Oei and Nieuwenhuijzen, 2005). Earranna *et. al.* (2010) isolated and identified *Bacillus pumillus* from contaminated spawns of oyster mushrooms, using morphological, biochemical and molecular approaches. Kumar *et al.* (2013) recorded the maximum contamination in spawn of *A. bisporus*, *P. florida* and *C. indica* prepared with bajra grains (20.66 per cent), followed by maize (14.33 per cent) and sorghum grain (12.66 per cent). They identified *Aspergillus* spp. as the most

prevalent fungal contaminant (3.00 to 6.00 per cent) followed by *Penicillium* spp. (2.33 to 4.00 per cent).

In the present study, spawns were prepared to attain the final moisture content of 60 per cent. Similarly, Dawit (1998) stated that water content of 40-60 per cent is optimal for spawn preparation. However, Tesfaw *et al.* (2015) recorded the optimum moisture content as 52 per cent. Higher water content accelerated mycelia growth, but increased the incidence of wet spot bacteria, whereas spawns prepared at 35 per cent moisture content slowed down the mycelia growth.

Amendment studies on spawns revealed that iron and thiamine recorded an inhibitory effect on mycelial growth of *Pleurotus* spp. Contrary to the present findings, Chang and Miles (2004) recommended the incorporation of vitamins *viz.*, biotin and thiamine into the spawn substrate. Kumar (2015) reported that spawns prepared from maize supplemented with gram husk had the longest economic cropping period for *A. bisporus* (75.33 days), *P. florida* (72.66 days) and *C. indica* (76.66 days) whereas, the amended spawn recorded minimum biological efficiency, compared to maize alone.

5.9. CULTIVATION

Mushrooms are one of the highest protein producers per unit area and time. Their production from agro wastes can be a very effective weapon in fighting malnutrition (Singh, 2011). *Pleurotus* species easily grow on lignocellulosic materials because they have high saprophytic characteristics. Jafarpour *et al.* (2011) reported that, using substrates containing a high content of protein and nitrogen can shorten the growth period and increase BE of oyster mushrooms.

P. cystidiosus (coremial) failed to produce significant fruiting bodies. This may be because basidiocarps of *P. cystidiosus* were collected in warm seasons (summer and early autumn) from living, not autochthonous trees and hence faced difficulties in the artificial cultivation of fruit bodies (Miller, 1972). Thus

multilocation trials screened out *P. cystidiosus* (non-coremial) and *P. opuntiae* as the best isolates, suited for the climatic conditions of Trivandrum and Idukki, respectively. Optimising type of substrate and growing conditions help in obtaining fruiting bodies with the best characteristics and yield; thus introducing these newer species into commercial cultivation (Dawidowicz and Siwulski, 2017).

The use of different types of substrate by fungus will depend on its capacity to secrete enzymes such as oxidative (ligninase, laccase, manganese peroxidase) and hydrolytic (cellulase, xylanase and tannase) enzymes which are involved in utilizing lignocellulosic substrates (Donini *et al.*, 2009). The substrates used for production of oyster mushroom are normally nitrogen deficient. Addition of organic and inorganic supplements to the substrate can improve the yield of mushroom and therefore been recommended by many workers (Menolli *et al.*, 2014). There is an optimal concentration of supplements which can support the mycelium growth (Han *et al.* 1981), whereas extreme amounts of supplements may decrease their effect.

Screening of different amendments revealed that, 4 per cent wheat bran was the best amendment for *P. cystidiosus* (non-coremial) in paddy straw and coirpith with BE of 125.52 and 37.57 per cent, respectively, whereas spraying of rubber wood sawdust with 2.5 per cent of 1 *M* KH_2PO_4 gave the maximum BE (192.76 per cent). In case of *P. opuntiae* wheat bran 4 per cent was the best amendment in paddy straw and coirpith (BE of 80.60 and 46.03 per cent, respectively), whereas the best amendments in rubber wood sawdust were either wheat bran 4 per cent or neem cake 4 per cent (BE of 91.37 and 91.38 per cent respectively). Screening of substrates and amendments revealed that spraying of rubber wood sawdust with 1 *M* KH_2PO_4 2.5 per cent was the best combination for *P. cystidiosus* (non-coremial), whereas rubber wood sawdust amended with either 4 per cent wheat bran or neem cake gave the maximum BE for *P. opuntiae*. In the

present study, sawdust of rubber tree was identified as the best substrate for *P. cystidiosus* (non-coremial) and *P. opuntiae*. This was because nutrient status of rubber sawdust is higher than other sawdust. It contains 1.68 per cent N, 0.48 per cent P, 1.18 per cent K, 0.12 per cent Ca and 0.04 per cent Mg. Also sawdust has uniform size and structure which facilitates enrichment of substrate (Sumi, 2016).

Similar to the present study Stamets (1993) recommended sterilized hardwood sawdust (maple, oak, beech or elm), pasteurized wheat and paddy straw as suitable substrates for the fruiting of *P. cystidiosus* with BE of 50-75per cent. Howlader *et al.* (2011) recorded the highest biological yield (196.3 g packet^{-1}), economic yield (189.0 g packet^{-1}), effective fruiting bodies (37.25), length of stipe (4.95 cm) and thickness of pileus (1.35 cm) for Pcys-1 followed by Pcys-2, Pcys-4 and Pcys-5. *P. cystidiosus* recorded slower rate of spawn running on the cotton seed hulls substrate due to the high C/N ratio whereas, slow growth and delayed formation of the fruiting body on perilla stalks substrate was caused by an excess of nitrogen (Yang *et al.*, 2013). Hoa *et al.* (2015) recorded yield of *P. cystidiosus* on first, second and third flush with 100 per cent sawdust as 77.76, 62.82 and 41.01 g bag^{-1} respectively, with BE of 36.27 per cent. Miah *et al.* (2017) recorded the highest number of fruiting body (57.20), weight of fruiting body (4.45 g), biological yield (227.68 g) and benefit cost ratio (4.25) of *P. ostreatus* with the sawdust of mahogony tree. *P. cystidiosus* strain AG55/4661 recorded biological efficiencies of 46 and 37 per cent in first flush and second flush respectively, with a cap diameter and stipe length/diameter of 7.0 ± 0.0 cm and 3.0 ± 0.0/0.8 ± 0.0 cm, respectively, at 20 ^{0}C and 9 h daylight (Shnyreva *et al.*, 2017).

Neopeat is the registered trade name of degraded, washed and sterilised coirpith obtained after processing coconut husk with a pH of 5.7 to 6.5 (Sumi, 2016). Study revealed very low yield of *P. opuntiae* and *P. cystidiosus* (non-coremial) in neopeat. This may be due to high content of fibrous material, lignin and tannin in coirpith (Priya *et al.*, 2017). Sumi (2016) revealed that *H. ulmarius* showed minimum BE in neopeat with maximum contamination (35 per cent) by

different pests like phorid flies, grubs of staphylinid beetles and spring tails. Priya *et al.* (2017) found that *Auricularia polytricha* failed to produce any fruiting bodies when neopeat was used as substrate. Contrary to the above findings, Mason (2003) recommended coirpith as a good substrate for mushroom cultivation as it is rich in cellulose and lignin, with good water holding capacity.

The substrates directly affected the time frame to attain the maximum mycelial growth, initiation of pin heads and yield attributes for oyster mushrooms (Islam *et al.,* 2017). In the present study, *P. cystidiosus* (non-coremial) and *P. opuntiae* took the minimum time for first harvest, pinhead formation and first harvest in paddy straw followed by rubber wood sawdust. The presence of right proportion of alpha-cellulose, hemicellulose and lignin was the probable cause of higher rate of mycelium running in paddy straw (Mondal *et al.*, 2010). Saw dust took the maximum time for spawn run due to presence of different kinds of polyphenolic substances (Wang, 1982) and low content of cellulose (Gohl, 1993). Similarly, Mondal *et al.* (2010) recorded the minimum time for spawn run, pinhead formation and first harvest on banana leaves & rice straw (1:1) whereas, maximum number of effective fruiting bodies was observed with sawdust.

Albores *et al.* (2006) noticed a positive correlation between the C/N ratio of substrate and mycelium growth rate. Yang *et al.* (2013) recorded that *P. ostreatus* on 80 per cent cotton seed hull with C/N ratio of 34.87 took longer time to complete the colonization period than rice straw 80 per cent and wheat straw 80 per cent with C/N ratios of 49.19 and 64.63 respectively.

Dias *et al.* (2003) recorded significant improvement in the production of *P. sajor-caju* when corn straw was supplemented with wheat flour, gypsum and calcium carbonate. Donini *et al.* (2009) identified cereal brans as important source of organic nitrogen for the growth of the mycelium mass improving its productiveness and BE. The quantity and the kind of bran may vary according to the species or the strain under development as well as its growth stage. In line with the present study, Moonmoon *et al.* (2010) recorded improved BE of

Lentinus edodes, when wheat bran 25 per cent was used as supplement compared to rice bran. Javed *et al.* (2012) and Apprich *et al.* (2014) found that substrates enriched with wheat bran required no further addition of nitrogen supplements. This may be because, wheat bran consisted of protein (12 per cent), carbohydrates (60 per cent), fat (3.5-3.9 per cent), minerals (2-8.1 per cent), vitamins and bioactive compounds. It also contained compounds such as carotenoids, lignans, phenolic acids, phytosterols, flavonoids, phytic acid and α-tocopherol. Contrary to the above findings, Cisarikova (2016) recorded that, beech wood sawdust enriched with 25 to 50 per cent wheat bran failed to produce sporocarps of *P. ostreatus* in bag method of cultivation.

Stoller (1940) proposed an organic fertilizer raw material for the cultivation of *P. ostreatus*. The fertilizer mixture consisted of 1-25 parts of vinegar residue, 2-25 parts of gypsum, 2-10 parts of ferrous sulfate, 5-25 parts of bran, 1-20 parts of urea, 1-12 parts of monopotassium phosphate, 1-20 parts of sucrose and 1-20 parts of cottonseed hull, with high nutrition absorption and BE.

In the present study, spraying of 2-4 per cent gypsum had an inhibitory effect on yield of *P. cystidiosus* (non-coremial) and *P. opuntiae*. Contradictory to the above findings, Oei (1996) reported that *Lentinula edodes* gave higher yield on wood chips supplemented with calcium carbonate and calcium sulphate. Royse (2003) reported that BE of *Lentinula edodes* was improved in substrates supplemented with 0.2, 0.4 and 0.6 per cent calcium carbonate. Khanagoudar and Mallesha (2017) recorded the maximum yield of *H. ulmarius* and *P. florida* in paddy straw (533.3 and 463.3 g, respectively) and coirpith (366.6 and 360.0 g, respectively) amended with calcium sulphate. This may be because calcium ions enhanced the production of fruiting bodies. Also, an increase in number of sporocarps of *Pleurotus* spp. was recorded on spraying the beds with GA. Similiarly, Eswaran and Ramabadran (2000) reported an increased number, weight of sporophores and BE when beds were treated with GA (100 ppm). The

stimulating action of GA might be due to increase in cell division and cell elongation. Evans (2005) reported that, GA had no significant effect on the growth and yield of mushrooms.

5.10. DEVELOPMENTAL MORPHOLOGY OF PLEUROTUS SPP.

The development of mushrooms can be divided in to two stages *viz.*, the vegetative and the reproductive stage. The transition from vegetative to reproductive stage is principally controlled by environmental conditions *viz.*, light, CO_2 concentration, temperature and humidity. Species of the genus *Pleurotus* perform a wide variety of morphological characters in different climates and environment which varies with the C/N ratio of the substrate (Estrada *et al.*, 2010).

In the present study, sporocarps of *P. cystidiosus* (coremial) and *P. cystidiosus* (non-coremial) took an average of five days from the day of pinhead formation to complete maturity whereas, sporocarps of *P. opuntiae* took an average of four days. Yasotha (2008) recorded the occurrence of very large, blackish brown fruiting bodies in a single bunch for *P. cystidiosus*, whereas *P. djamor* produced pink coloured fruiting bodies. Hoa *et al.* (2015) recorded the time for complete colonization, first harvest and total harvesting period of *P. cystidiosus* in substrates containing 100 per cent corn cob as 35.08 to 40, 64.24 and 42.40 to 48.57 days. Barrales and Mata (2016) recorded the formation of primordia in *P. opuntiae* 30 days after incubation, with an average of 472.5 g of sporocarps per bag. They noticed the production of white, large, leathery and scattered basidiocarps.

Contradictory to the above findings, Dahmardeh *et al.* (2010) reported slower mycelial growth and colonization of oyster mushrooms *i.e.* around three weeks, with appearance of fruiting bodies after 2 to 3 days.

5.11. PESTS AND DISEASES

Trichoderma sp., *Coprinus* spp. and Staphylinid beetles (*Scaphisoma* sp.), were the common contaminants observed for *P. cystidiosus* (non-coremial) and *P. opuntiae*, with paddy straw, rubber wood sawdust and neopeat. Addition of supplements increased the risk of contamination. This was because supplements provided good nutrients to other microorganisms (Yildiz *et al.*, 2002). Gahukar (2014) reported that two species of staphylinid beetles *viz.*, *Scaphisomanigro fasciatus* Champion and *Scaphisomanigro tetrastictum* Champion, feed on sporocarps, resulting in undeveloped pinheads in oyster mushrooms. Similar to the present findings, Priya *et al.* (2017) recorded the major insect pests from the beds of *Auricularia* spp. as phorid flies (*Megaselia* spp.) springtails (*Seira* spp.), staphylinid beetles and mites. Also major contaminants *viz.*, *Coprinus* spp., *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp. were recorded.

5.12. COMPARATIVE PERFORMANCE

Comparative performance of the new isolates *viz.*, *P. cystidiosus* (non-coremial) and *P. opuntiae* with the popular oyster mushrooms *viz.*, *P. florida* and *P. eous* respectively is an essential prerequisite towards introduction and commercialization of two new isolates.

5.12.1. Spawn production

Comparative spawn production studies revealed that minimum time for spawn run was recorded with *P. cystidiosus* (non-coremial) in paddy grains (amended with yeast 1 per cent) (5.61 days) and *P. opuntiae* in sorghum grains (amended with yeast 1 per cent) (9.28 days). Saha *et al.* (2012) recorded that *P. eous* took the minimum time for spawn run with wheat grains (9.66 days) followed by paddy grains (11 days), sorghum (11 days) and maize grains (11.33 days). Murugaiyan *et al.* (2017) reported that *P. florida* took the minimum time for spawn run (10 days) and pinhead formation (13 days) with maximum stalk

length, pileus diameter, pileus thickness and BE (76.76 per cent) when paddy grains and paddy straw was used as substrate for spawn production and cultivation respectively.

5.12.2. Mushroom Production

Comparative mushroom production studies revealed that cultivation of *P. cystidiosus* (non-coremial) on rubber wood sawdust sprayed with 1*M* KH_2PO_4 2.5 per cent recorded the maximum crop period (145.30 days), weight of sporocarps (35.80 g), number of sporocarps (106.90), total yield from three harvest (3858.60 g) and BE (192.2 per cent), whereas, *P. opuntiae* in rubber wood sawdust amended with wheat bran 4 per cent gave the maximum number of sporocarps (137.60 days), total yield from three harvest (913.60 g) and BE (91.36 per cent). Mondal *et al.* (2010) recorded that effective fruiting bodies of *P. florida* ranged from 8.5 to 37.25 per bunch and it varied with the type of substrate used for cultivation. *P. pulmonarius* took an average of 22 days for spawn run in rice straw substrate compared to 35 days in sawdust substrate (Akinmusire *et al.*, 2011). Musieba *et al.* (2012) reported that, *P. citrinopileatus* took 13 days to colonize 1 kg rice straw substrate compared to 21 days in sawdust substrate. Subramanian *et al.* (2014) recorded that *P. eous* took 7.33, 9.66, 11.83, 15.33 and 17.83 days for spawn run, first harvest, pin headed appearance, second harvest and third harvest with alternate layer spawning method and paddy straw as substrate. Obodai *et al.* (2014) identified significant variation in the BE among seven species of *Pleurotus* spp. grown on sawdust, due to difference in the species/strains of mushroom used, composition/formulation of the substrates and ambient environmental conditions (temperature and relative humidity). Mishra *et al.* (2015) recorded that *P. florida* produced milky white sporocarps of size 6.7 cm diameter and 12.5 cm length with wheat straw as substrate. They recorded number of fruiting bodies, yield and biological efficiency as 96.2, 531.7 g/kg of substrate and 53.17per cent,

respectively. Naraian and Dixit (2017) recorded the highest BE for *P. florida* (90 per cent) followed by *P. sajur-caju* (89 per cent) and *P. eous* (82 per cent).

Dawit (1998) stated that supplements change physical conditions of substrates making it suitable for cultivation of mushrooms. Onyango and Palapala (2011) noted that supplementation of substrate with bran provided a protein rich medium which increased the rate of mycelial growth by two fold. *P. eous* recorded minimum time for spawn run (12.66 days) and maximum BE (96.05 per cent) when, wheat straw was supplemented with 2per cent pegionpea bran followed by chickpea bran (13.33 day, 84.65 per cent) and soybean flour (15 days, 82.87 per cent).

5.12.3. Seasonal variation in production of *P. cystidiosus* (non-coremial) and *P. opuntiae*

The sporophores of *P. cystidiosus* (non-coremial) in Idukki were larger, white and very attractive, yielding 1927.20 g from a single sawdust bed compared to very low yield in Vellayani condition. Contradictorily, the performance of *P. opuntiae* in Idukki was very low and the sporophores were small, colour varied, yielding 712.28 g from a single sawdust bed (Table 30). Thus, the study recommends *P. cystidiosus* (non-coremial) as the best oyster mushroom species for low temperature areas like Idukki, Munnar and Wayanad, whereas, *P. opuntiae* for high temperature regions. Thus cultivation trials with *P. cystidiosus* (non-coremial) and *P. opuntiae* were carried out in Idukki (Thankamani) and Trivandrum (Vellayani) respectively. In Idukki, *P. cystidiosus* (non-coremial) recorded the maximum yield during June-September (1927.20 g) followed by October-January (1875.28 g) and February-May (1457.55 g). In Vellayani, *P. opuntiae* recorded the maximum yield during June-September (913.40 g) followed by February-May (811.82 g) and October-January (689.18 g) (Fig 7). Uddin *et al.* (2011) suggested that the environmental conditions of Bangladesh during winter months (December to February) in the temperature zone of 14-27 ^{0}C with relative humidity 70-80 per cent was most suited for the cultivation of oyster mushrooms

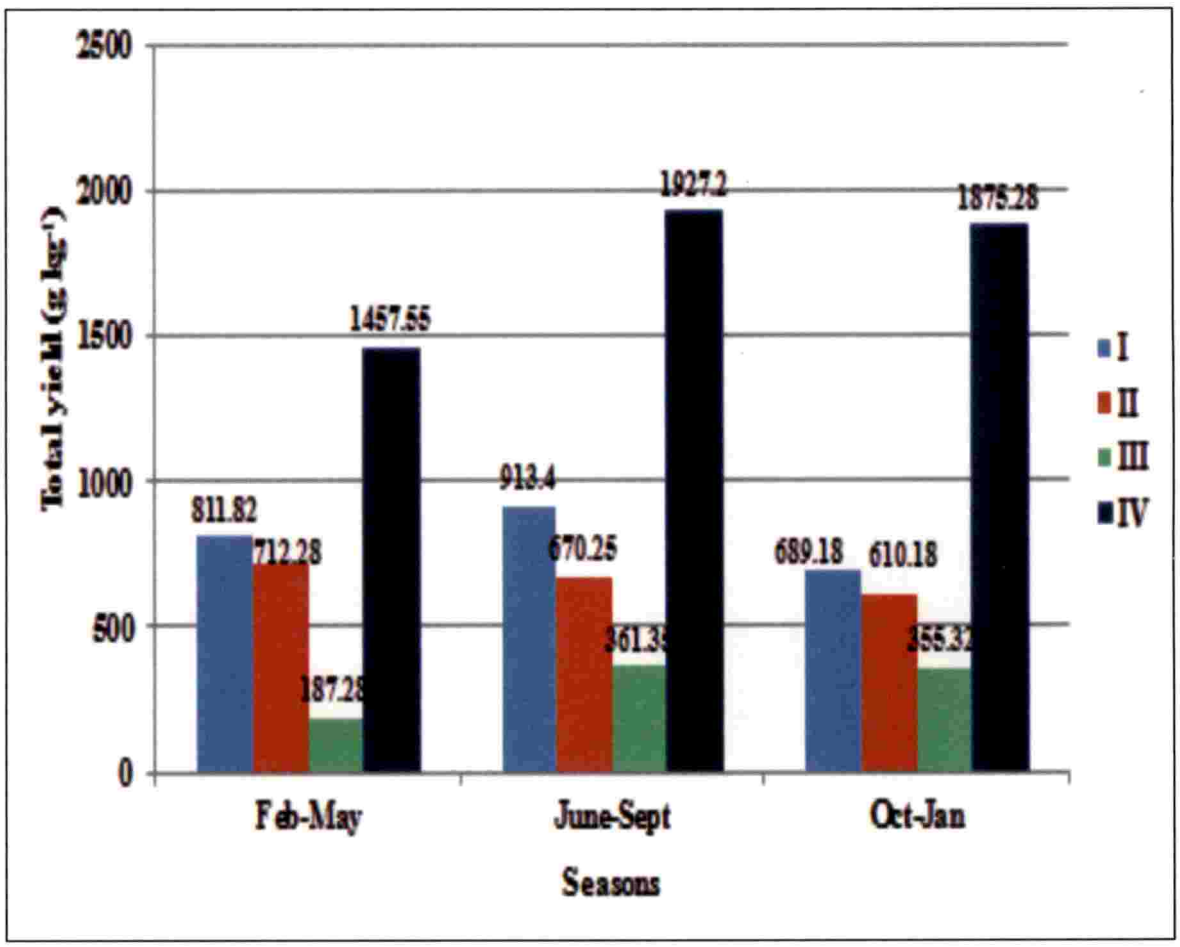


Fig 7. Seasonal variation in production of *P. opuntiae* and *P. cystidiosus* (non-coremial) in Vellayani and Idukki, I: *P. opuntiae* in Vellayani, II: *P. opuntiae* in Idukki, III: *P. cystidiosus* (non-coremial) in Vellayani, IV: *P. cystidiosus* (non-coremial) in Idukki.

viz., *P. ostreatus*, *P. florida*, *P. sajor-caju* and *Pleurotus high king*, while August to October was unfavorable. AICRP (2013) carried out multilocation trials with six high yielding strains of *Pleurotus* species, at 12 different centres. The study recorded maximum yield with strain Pl-12-05 (122.6 kg 100 kg^{-1} of dry substrate) at Udaipur Centre followed by strain Pl-12-02 (122.5 kg 100 kg^{-1} of dry substrate) at Raipur centre.

5.13. CROPPING CONDITIONS

Different environmental factors affect the fruiting body development of mushrooms (Khan and Chandra, 2017). In the present study, mushroom beds were kept in mushroom house where favourable climatic conditions were maintained. Moisture plays an important role in the growth and development of oyster mushrooms. Hence water was sprayed three to four times a day on the beds of *Pleurotus* spp. in order to maintain temperature (25±2 ^{0}C) and RH (more than 80 per cent) for inducing the production of fruiting bodies. Intensity, duration, and wavelength of light are vital components of any treatment. Light positively affects both aggregation of hyphae and maturation of the fruiting body, whereas, the formation of the hyphal knot can be suppressed by the light. Light is required for the normal expansion of the pileus and formation of spores, whereas no light is required for the spawn run phase (Zadrazil and Kurtzman, 1982). Kues and Liu (2000) reported that a light pulse of low energy is sufficient to initiate aggregation in hyphae.

The carbon dioxide content of the atmosphere have an intense impact on sporophore development, *i.e.* higher concentration activate growth of mycelia, whereas fruiting bodies get deformed at lower concentrations, with lengthened stipe and reduced pileus (Scrase and Elliott, 1998). Milkwood (2004) recorded normal sporophore development of *A. bisporus* at 0.2 per cent carbon dioxide concentration whereas, concentration above 1-5 per cent activated elongation of stipe and prevented expansion of cap.

High humidity content around 90-95 per cent is essential for pinning and fruiting of mushrooms. Development of sporophore occurs within a range of temperature, *i.e. A. bisporus* from 16-18 ^{0}C, *Coprinus cinereus* from 25-28 ^{0}C and *Flamulina velutipes* at 18 ^{0}C (Kues and Liu, 2000). Oei and Nieuwenhuijzen (2005) recorded that *P. abalonus and P. djamor* required temperatures of 15-35, 12-18 & 18-25, 20-30 & 24-30 and 25-30 & 20-30 for incubation, induction, fructification and harvest respectively. Khan and Chandra (2017) noticed that, *P. cystidiosus* recorded very thin spawn growth at 16 and 36^{0}C, whereas no growth was recorded beyond 8 day of inoculation at 36 ^{0}C.

5.14. PROXIMATE CONSTITUENTS OF PLEUROTUS SPP.

Nutrient composition of mushrooms varies depending upon the species and composition of growing substrate (Goyal and Grewal, 2006). The genus *Pleurotus* comprises various edible mushroom species, with important medical and biotechnological properties (Nelson *et al.,* 2010). Hence, considered as balanced food stuff, with good source of proteins, vitamins, fats, carbohydrates, amino acids and minerals (Kumari *et al.*, 2011; Ziarati and Shanderman, 2014).

Mushrooms generally have high moisture content which accounts for their short shelf life, promoting susceptibility to microbial growth and enzyme activity (Adebayo *et al.*, 2011). Fresh mushroom contains 90 per cent moisture and 10 per cent dry matter, whereas, dry mushroom contains 90 per cent dry matter and 10 per cent moisture (Johnsy and Davidson 2011). Among the four isolates, highest moisture content was recorded for PNC1 (94.05 per cent) which is in agreement with those reported by other workers (Shashirekha *et al.,* 2005; Jaworska and Bernas, 2011). Zhang *et al.* (2007) recorded the moisture content of *P. opuntiae* as 90.70 per cent. Khan *et al.* (2008) recorded a moisture content of 85-88 per cent for fresh oyster mushrooms. Moisture content is influenced by age and strain of mushrooms, growing environments and postharvest environments (Ahmed *et al.*, 2013).

Carbohydrate content of mushroom represents the bulk of fruiting bodies accounting for 50 to 65 per cent on dry weight basis, with around 11 per cent free sugars (Thatoi and Singdevsachan, 2014). Here, *P. florida* showed the maximum carbohydrate content (64.94 mg g^{-1}), followed by *P. cystidiosus* (non-coremial) (58.55 mg g^{-1}). Naraian and Dixit (2017) recorded the highest carbohydrate content in *P. eryngii* (41 g) followed by *P. sajor-caju* (38 g) and *P. florida* (34 g).

Among the bioactive compounds in mushrooms, polysaccharides show antitumoral, antiviral and immunomodulatory activities (Mizuno and Nishitani, 2013). Reducing sugars, non-reducing sugars and total sugars of mushrooms contribute for the expansion of fruiting bodies and determine their nutritional quality. In the present study, *P. cystidiosus* (non-coremial) recorded the maximum starch content (200.55 mg/g) whereas, *P. florida* recorded the maximum cellulose content (60.4 mg/g). Gupta and Kapoor (1990) recorded the highest percentage of reducing sugars in *Volvariella volvacea* (18.0 per cent) followed by *P. cystidiosus* (16.5 per cent) whereas, the highest percentage of non-reducing sugars was found in *P. cystidiosus* (3.6 per cent) followed by *P. sajor-caju* (3.2 per cent). Bora and Kawatra (2014) recorded the total soluble sugars, reducing sugar and non-reducing sugars of *P. florida* as 3.01, 0.38 and 2.63 per cent respectively. Jahangir *et al.* (2015) recorded total soluble solids, reducing sugar, non reducing sugar and total sugar contents of *P. ostreatus* as 3.66 Brix, 3.41 per cent, 6.43 per cent and 9.84 per cent respectively. Adedokun and David (2016) reported that starch content of *P. floridanus* recorded the highest value when sawdust was used as substrate (5.31 g 100 g^{-1}).

Crude protein is found in high levels in edible mushrooms and varied between 15.2 g/100 g dried weight in *L. edodes* to 80.93 g 100 g^{-1} dried weight in *Agaricus bisporus*. (Barros *et al.*, 2008). Also bioactive proteins *viz.*, ribosome inactivating proteins, lectins, antifungal proteins, ubiquitin-like and ribonucleases

proteins, fungal immunomodulatory proteins and other enzymes have been isolated and characterized. In the current study, *P. cystidiosus* (non-coremial) recorded the maximum protein content (30.20 mg g^{-1}) followed by *P. eous* (21.98 mg g^{-1}). Bernas (2006) stated that differences in protein contents could be due to species/strain, growth substrate, size of pileus, time of harvest and level of nitrogen availability in the substrate. Das *et al.* (2014) recorded the highest protein content in *P. ostreatus* (856 µg/g), followed by *P. floridanus* (448 µg g^{-1}), *P. pulmonarius* (417 µg g^{-1}) and *P. florida* (294 µg g^{-1}).

Fat content in mushrooms is relatively low however, mushroom is rich in essential unsaturated fatty acids. Here, *P. cystidiosus* (non-coremial) recorded the maximum fat content (4.25 per cent), followed by PO1 (2.25 per cent). Akyuz and Kirbag (2010) recorded fat content of *P. ostreatus*, *P. sajor-caju* and *A. bisporus* as 0.9, 1 and 0.8 per cent dry weight, respectively. Mushroom contains good quality dietary fibre, both soluble and insoluble which helps in lowering the cholesterol. The soluble fibre is mainly composed of β-glucans and chitosans, which are components of the cell walls (Kakon *et al.* 2012). In the present study, *P. florida* showed the highest fibre content (10.49 per cent) followed by *P. eous* (10.11 per cent). The result is consistent with the values reported by Atri *et al.* (2013) who recorded that, dietary fibre (mainly chitin) in *Pleurotus* spp. ranged from 10 to 31 g per 100 g of dry weight.

Analysis of ash content among the three isolates revealed that *P. florida* recorded the highest ash content (10.65 per cent). Similar to the present findings, Khan *et al.* (2008) recorded the highest ash content in *P. florida* (8.3 g $100g^{-1}$ dry sample) followed by *P. sajor-caju* (8 g/100g dry sample), *P. ostreatus* (7.4 g $100g^{-1}$ dry sample) and *P. cystidiosus* (7.4 g $100g^{-1}$ dry sample). Das *et al.* (2014) reported that *P. florida* has the highest percentage of ash (10.6per cent), followed by *P. ostreatus* (8.7 per cent), *P. floridanus* (6.1 per cent), *P. flabellatus* (5.9 per cent) and *P. pulmonarius* (4.8 per cent).

The antioxidant value of mushrooms is comparable with that of foods of vegetable origin. The compounds responsible for the antioxidant power in mushrooms are selenium, phenolic compounds, ergothioneine, tocopherols and carotenoids. Thus a good number of edible mushrooms could be used as natural antioxidants for their high potential against oxidative stress (Palacios *et al.*, 2011; Liu *et al.*, 2012; Reis *et al.*, 2012). Here *P. cystidiosus* (non-coremial) recorded the maximum values of antioxidants (485.45 µg Eq.g^{-1} of ascorbic acid)), followed by *P. opuntiae* (370.55 µg Eq.g^{-1} of ascorbic acid). Hoa *et al.* (2017) reported that, fruiting body extracts of *P. ostreatus* and *P. cystidiosus* showed the highest values of DPPH free radical scavenging ability (4.44, 4.02 mg ml^{-1}), with the lowest values of IC50 (3.55, 2.4 mg ml^{-1}).

β-carotene is a naturally occurring carotenoid with powerful antioxidant activity. *P. cystidiosus* (non-coremial) recorded the maximum beta carotene content (25.69 µg $100mg^{-1}$), followed by *P. eous* (19.82 µg $100mg^{-1}$). Nakalembe *et al.* (2015) recorded low beta carotene values (12.60-17.70 µg g^{-1} dry weight) for *Termitomyces microcarpus*, *Termitomyces tyleranus*, *Termitomyces clypeatus*, *Volvariella speciosa* and *Polyporus tenucuilus.*

Polyphenol content is the most important component imparting antioxidant quality of mushrooms and have the capacity to eliminate free radicals due to presence of hydroxyl group (Ferreira *et al.*, 2009). In the present study, *P. cystidiosus* (non-coremial) had the maximum polyphenol content (7.55 mg g^{-1}), followed by PO1 (7.14 mg g^{-1}). Hoa *et al.* (2017) recorded high phenolic content (37.53 mg GAE g^{-1}) in *P. cystidious* with corn cob 100 per cent as cultivation substrate. *P. cystidiosus* (non-coremial) recorded the highest antioxidant activity due to the presence of beta carotene and phenolic content (Hoa *et al.*, 2017).

Oyster mushrooms are low in calories due to limited content of lipids. In the present study, *P. cystidiosus* (non-coremial) had the maximum energy value (359.45 Kcal) followed by *P. florida* (337.89 Kcal). The result is in agreement with the observation of Barros *et al.* (2008) who reported differences in caloric

value among different species of wild and commercial mushrooms. Khan *et al.* (2008) recorded the highest metabolizable energy in *P. cystidiosus* (262.8 kcal $100g^{-1}$) followed by *P. sajor-caju* (254.1 kcal $100g^{-1}$), *P. geestaranus* (252.7 kcal $100g^{-1}$) and *P. florida* (250.1 kcal $100g^{-1}$). The variation on the per cent moisture, nitrogen, crude protein and ash may be due to the geographical location, time of harvesting and stage of collection (Bambhaneeya *et al.*, 2015). Madrupji (2017) recorded that *P. cystidiosus* had 11.56 per cent crude protein, 65.83 per cent moisture, 1.85 per cent nitrogen and 1.7 per cent ash, whereas, *P. populinus* had 14.50 per cent crude protein, 83.42 per cent moisture, 2.32 per cent nitrogen and 4.11 per cent ash. Vishwakarma *et al.* (2017) recorded ash, moisture, phenolic and fibre content of *P. cystidiosus, P. flabellatus, P. florida* and *P. ostreatus* in the range of 5.72 to 7.69 per cent, 80.29 to 85.18 per cent, 37.70 to 39.01 mg g^{-1} and 11.42 to18.08 per cent, respectively.

5.15. ANTICANCEROUS ACTIVITIES OF *PLEUROTUS* SPP.

Besides the taste and nutritional value, mushrooms also have therapeutic properties like anti-inflammatory, immunomodulatory, anti-cancer and ribonuclease activities (Wang *et al.*, 2005). Cancer is a leading cause of death worldwide. Many management options for cancer are available *viz.*, surgery, chemotherapy, radiation therapy and palliative care. Treatment options often are expensive and have side effects. This situation has resulted in the search for new antitumor substances from various natural sources for effective and safer control (Chung *et al.,* 2010). The bioactive compounds of mushrooms can complement classical cancer therapy and counter the side effects of cancer such as nausea, bone marrow suppression, anaemia etc. The MTT or in vitro cell proliferation assay is one of the most widely used assay for evaluating preliminary anticancer activity of both synthetic derivatives and natural products. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines and gives an indication of whole cell cytotoxicity (Cauley *et al.*, 2013).

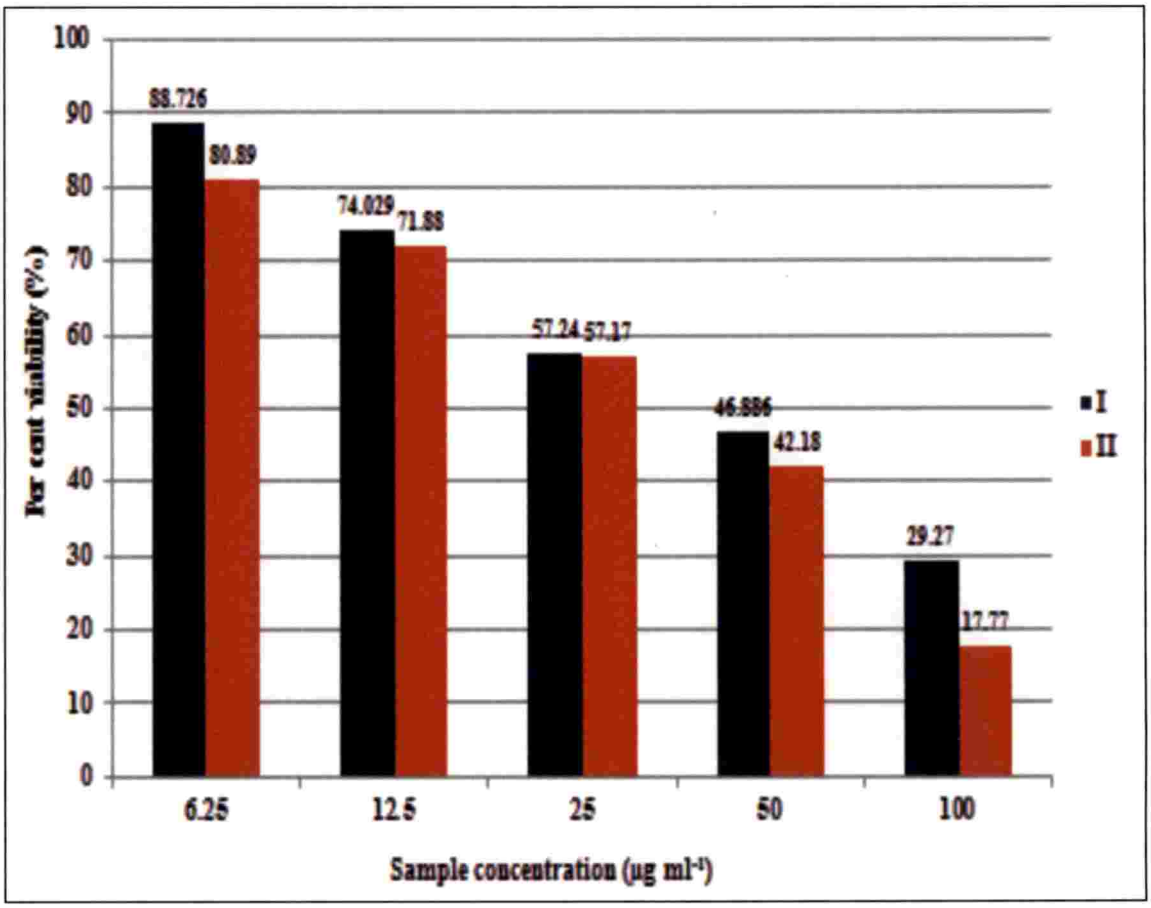


Fig 8. Invitro anti-proliferative activity of *Pleurotus* spp. against colon cancer cell line (HeLa), I: *P. cystidiosus* (non-coremial), II: *P. opuntiae*

MTT assay revealed that, dried extracts of *P. cystidiosus* (non-coremial) and *P. opuntiae* at concentration of 100 µg/ml recorded the highest anti-proliferative effect on colon cancer cells, with minimum cell viability of 45.99 and 29.27 per cent, respectively (Fig 8). Direct microscopic observations revealed indications of cytotoxicity with LD-50 values of 78.29 and 55.039 µg ml^{-1} respectively. The results of the current work are consistent with the studies demonstrating that, basidiomycetes have antitumor activity by direct or indirect mechanisms (Talarico *et al.*, 2005). A possible explanation of observed cytotoxic effects lies in the fact that, polysaccharide extracts of various mushrooms are highly active in the generation of reactive oxygen species (ROS) (Durgo *et al.*, 2013). Although cancer therapies can inhibit tumour growth and prolong the life span of humans, they usually compromise the immune system and damage genetic material of healthy cells. Thus, supportive biotherapy, using the *Pleurotus spp.* extracts, is a promising alternative/supplement to conventional cancer therapies.

5.16. KEEPING QUALITY OF *PLEUROTUS* SPP.

Various physiological and morphological changes occur after harvest, which make the mushrooms unpalatable for consumption (Kelshrewtha *et al.*, 2009). Mushrooms are extremely perishable in nature and cannot be kept for more than one day after harvesting at ambient conditions. Also their shelf-life is limited to a few days under normal refrigeration conditions, which is a constraint on the distribution and marketing of fresh product, making extension of mushroom's shelf life a constant quest (Kalac, 2013).

In the present study, harvested mushrooms of *P. opuntiae*, *P. florida, P. cystidiosus* (non-coremial) and *P. eous* recorded shelf life of 24, 24, 48 and 24 h respectively in natural condition, whereas 3, 3, 5 and 3 days were recorded when preserved in refrigerated condition. The shelf life of mushrooms is more in refrigerated condition due to the fact that, low temperature retards the growth of microorganisms, decreases the rate of postharvest metabolic activities of the

mushroom tissues and minimizes the moisture loss (Rai and Arumuganathan, 2008).

Choi and Kim (2003) reported that, shelf life of *P. ostreatus* packed with polyethylene film (60 mm thickness) was 8-11 days at 0 ^{0}C, 4-6 days at 5 ^{0}C, 2-3 days at 10 ^{0}C and 1-2 days at 20 ^{0}C. During storage, film packaging retarded the deterioration of appearance and texture of mushrooms. *P. ostreatus* under modified atmosphere packaging (MAP) using PE 65S Cryovac (C), PD 961 Cryovac (D) films and low-density polyethylene (LDPE) (Maria *et al.*, 2009) recorded improved shelf life. The active MAP composed of 1.5 per cent oxygen and 20 per cent carbon dioxide which helps in maintaining the quality and shelf-life of the mushroom. Pandey *et al.* (2016) recorded that, *P. cystidiosus* recorded the maximum shelf life of 3-4 and 20-30 days when stored in trays wrapped with antifog film at 27-29 ^{0}C and 4-6 ^{0}C respectively, followed by *P. djamor* (1-2 days and 4-6 days) and white *Pleurotus* spp (1-1.5 and 3-5 days). Sumi (2016) reported that, *H. ulmarius* packed in perforated polythene bags could be kept fresh for eight hours in normal atmospheric condition and up to five days in refrigerated condition, whereas, *P. florida* could be kept for six hours and three days respectively. Priya *et al.* (2017) reported that fresh samples of *Auricularia polytricha* remained fresh for 24 h under normal atmospheric conditions, whereas under refrigerated conditions (4 ^{0}C) could be kept fresh for three days without any remarkable changes.

5.17. SENSORY EVALUATION

Mushrooms have been used as food for centuries because of their unique taste, flavours, textures, nutritional and medicinal attributes (Cheung, 2010). Mushrooms have a characteristic taste, known as umami (delicious savoury in Japanese) making them flavourful and versatile for using in various culinary dishes. Value added products prepared from mushrooms are developed by small to medium scale farmers who do their own processing and sell directly to customers through farmers markets, individual and direct wholesale orders (Hoa and Wang, 2015). Assessment of cooking quality and sensory evaluation revealed

that, masala curry prepared from *P. cystidiosus* (non-coremial) recorded the maximum values for appearance (9.1), colour (9.3), flavor (9.0), texture (9.0), taste (9.2), aroma (9.1) and overall acceptability (9.4) among all other recipes of *Pleurotus* spp. studied. Similar to the present finding, Gateri *et al.* (2015) reported that, cream white colour, taste and flavour of *P. abalonus* was more superior in terms of appearance, taste, flavour and overall acceptability than the white sporocarps of *P. florida* for both cooked and uncooked samples. This may be because of the presence of 54 number of volatile oils *viz.,* palmitic acid, indole and myristic acid; and 16 numbers of aromtic components (Usami *et al.*, 2014). Similar results on product development and evaluation coincided with those reported by Priya *et al.* (2017); and Bora & Kawatra (2014).

Stamets (1993) reported that *P. cystidiosus* has oyster like taste but of vegetable nature. Rosli *et al.* (2012) reported that, potato pudding fortified with 5 per cent powder of *P. sajor-caju* improved the nutrient contents and sensory qualities of carbohydrate-based products. Lu *et al.* (2014) standardised the preparation of sausage for *P. nebrodensis* and *P. ostreatus*, based on sensory evaluation and textural properties. Kaur (2016) reported that, mushroom lemon soup powder prepared from *P. florida* recorded the optimum overall acceptability value up to six months of storage. Mishra *et al.* (2018) reported that mushroom pakories prepared from *P. sajor-caju* recorded the maximum oraganoleptic value (8.72), followed by mushroom pulav (8.31) and mushroom potato curry (8.23).

5.18. POST HARVEST TECHNOLOGY OF PLEUROTUS SPP

Pretreatment is necessary to check the discolouration during processing of mushrooms (Pruthi, 1978). Deshpande and Tamhane (1981) recorded that dried mushrooms without pretreatments resulted in undesirable, hard textured product, which crumbled readily. Pre-treatments of mushrooms before drying in one form or other *viz,* blanching, washing in water, KMS, CA, sugar, salt either alone or in combination help in checking enzymatic browning, stabilizing colour, enhancing flavour retention and maintaining textural properties (Singh *et al.,* 2007). The

differences among treatments may be attributed to difference in the bound moisture content, developed during the treatments (Suguna *et al.,* 1995).

Samples of *P. cystidiosus* (non-coremial) and *P. opuntiae* subjected to solar drying recorded the maximum contamination. Similar to the current findings, Lakshmipathy *et al.* (2013) reported that, open sun dried mushrooms (18 h of drying) recorded a significant higher number of microorganisms (7.78 log cfu ml^{-1}), than other dehydrated mushrooms. On the contrary, Kaur (2016) reported that, solar drying was the best drying technique, as it gave best results up to three months of storage and gave the maximum nutritional values, followed by cabinet drying. Tolera and Abe (2017) reported that treatment of *P. ostreatus* with 5 per cent salt solution followed by solar drying were advantageous in terms of product quality when compared to oven drying methods.

Evaluation of post-harvest treatments revealed that, CA treatment (1 per cent) followed by mechanical drying was the best treatment for *P. cystidiosus* (non-coremial) and *P. opuntiae*. This may be because CA has preservative action which prevents the activity of peroxidase and catalase enzymes thus preventing darkening. Arumuganthan *et al.* (2005) observed higher whiteness in *A. bisporus* powder treated with citric acid. Jyothi (2013) recomended CA treatment followed by solar drying as the most effective post harvest technique for producing highly acceptable dehydrated mushrooms. Kaur (2016) reported that, *P. eryngii* treated with citric acid (500 ppm) gave better colour during solar and cabinet drying.

Soaking of mushrooms in KMS followed by mechanical drying and powdering was the second best treatment for *P. opuntiae*. This may be because sulphuring or sulphating, using KMS prevents the enzyme catalyzed oxidative changes, inhibits microbial deterioration and withstands higher temperatures of drying (Kumar *et al.*, 2012). Kaur (2016) recorded that mushroom samples of *C. indica* and *P. eryngii* dried after pretreatment with KMS recorded the highest sensory evaluation scores for colour, texture and flavour compared to untreated sample. Singh *et al.* (2001) reported that fresh mushrooms of *A. bisporus* treated

with 0.5 per cent calcium chloride, 0.5 per cent KMS and 0.5 per cent sodium chloride; and packed in polypropylene retained the quality up to 3 days of storage at both ambient and refrigerated conditions.

5.19. STRAIN IMPROVEMENT OF *PLEUROTUS* SPP.

Somatic hybridization through protoplast fusion is a useful technique that facilitates recombination of economically important traits by harnessing the natural genetic diversity from phylogenetically distant species (Hodge *et al.*, 2010).

5.19.1. Barrage reaction

Compatibility studies revealed the occurrence of barrage reaction between the crosses *viz.*, *P. cystidiosus* (coremial) x *P. opuntiae* and *P. cystidiosus* (non-coremial) x *P. opuntiae.* Similar to the present findings, Esser and Blaich (1994) confirmed the compatibility between *P. ostreatus* and *P. cornucopias* through barrage reaction. Eyini *et al.* (2006) reported that *P. pulmonarius* and *P. florida* showed compatibility in dual culture plates with a thick barrage of intermingled hyphae at the zone of contact. Gharehaghaji *et al.* (2007) reported three types of incompatible reactions *viz.*, inhibition, *i.e.* one of the mycelia grows fast and surrounds other mycelium without any clamp connection, mutual repulsion *i.e.* formation of a line of demarcation, mutual intermingling and inhibition, *i.e.* two types of mycelia grow into each other and intermingle, followed by an antagonistic, lethal fusions without any clamp connection. Also mycelial development varied within the interaction zone, which ranged from appressed, fluffy to cottony. Rosnina (2017) reported a positive mating reaction between *P. pulmonarius* and *P. citrinopileatus*, identified by a flat and smooth mycelial mat at their junction zone.

5.19.2. Biochemical markers

A critical step in the development of an efficient protoplast fusion system for any organism is the availability of selectable markers which allows positive selection of hybrids/fusants. Nutritional complementation between auxotrophic

mutants is the most popular method for selecting the fusion products in microbial protoplast fusion (Chung *et al.*, 2010).

In the present study, double selection strategy was carried out to regenerate selectively the hybrid protoplasts between *P. cystidiosus* (non-coremial) and *P. opuntiae*, on the basis of complementation in media. Thus, dual biochemical markers *viz.*, carbendazim (1 m*M*) and vanillin (0.05 per cent) were selected. Here, hybrid protoplasts could regenerate and develop colonies due to complementation of either both the parental genome or neucleo-cytoplasmic interaction. As it showed biparental morphology, it could be a recombinant as suggested by Lalithakumari (2000). In line with the present study Eynii *et al.* (2006) selected the stable fusant strain of *P. pulmonarius* and *P. florida* by using biochemical markers *i.e.* carbendazim tolerance and utilization of the lignin degradation product, vanillin. Singh *et al.* (2007) identified the antifungals *viz.*, 210 μg ml^{-1} griseofulvin and 10 μg ml^{-1} clotrimazole as markers for the development of fusants between isolate H-13 of *A. bisporus* ITCC and isolate H-15 of *A. bitorquis* ITCC. Chakraborty and Sikdar (2010) screened hybrid strains between *C. indica* var. APK2 and *Pleurotus florida* based on the differential tolerance of sodium chloride level by the two parental genera. Kaur and Kapoor (2014) stated that double selection markers *viz.*, 2m*M* vanillin and 1000 ppm carbendazim ensured the selection of hybrids between *P. florida* PF-5 (MTCC 1801) and *P. sajor-caju* PSC3 (ATCC 62887) and eliminated any chances of selection of parent cultures.

5.19.3. Protoplast isolation

The release of protoplasts depends on three major factors: the lytic enzyme used, the osmotic stabilizer, physiological status of the organisms and complex interactions which exist between them.

5.19.3.1. Effect of Mycelium Age on Protoplasts Formation

Age of mycelium strongly influenced the protoplast yields as previously reported by Kelkar *et al.* (1990). Maximum release of protoplasts from *P. cystidiosus* (non-coremial) and *P. opuntiae*, was recorded from 4 (5.85×10^7 ml^{-1}) and 3 days old mycelium (5.93×10^7 ml^{-1}) respectively. This fact would be related to the changes in the hyphal wall, which passing from the exponential phase of growth becomes more resistant to enzyme degradation. It might be also associated with the high number of growing tips when young and actively growing cultures are used for protoplast production (Farina *et al.*, 2004). Parani and Eyini (2010) identified that 3 days old cultures of *P. eous* and *P. flabellatus* gave the maximum release of protoplasts.

5.19.3.2. Effects of Enzymes on Protoplast Isolation

Most fungi have a cell wall consisting largely of chitin and other polysaccharides and the plasma membrane is surrounded by three layers of cell wall materials, therefore, the enzyme consortium should comprise at least the major mycolytic enzymes, which are able to degrade the chitin and other components of the fungal cell wall. Protoplast yield from *P. cystidiosus* (non-coremial) and *P. opuntiae* was standardised at 30 mg ml^{-1} of a consortium of commercial enzymes *viz.*, β-glucanase, cellulase, protease, and chitinase named as the Lysing enzyme from *Trichoderma harzianum* (L1412 SIGMA Glucanex-lyophilized powder). Similarly Dhitaphichit and Pornsuriya (2005) used 9 mg Lysing Enzyme (Sigma L-1412) in 1 ml of 0.05 M sodium maleate buffer containing 0.6 *M* Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), pH 5 for isolation of protoplasts of *P. djamor* and *P. ostreatus*.

Curragh *et al.* (1992) suggested that optimal biomass: enzyme ratio needs to be investigated for each fungal strain, because an increase in ratio does not yield higher numbers of protoplasts. Djajanegara and Masduki (2010) used a mixture of cellulase Onozuka R-10 and macerozyme R-10 (1 per cent each) for *P. florida* and 2 per cent lyzing enzyme for *P. cystidiosus;* and obtained protoplast

yields of 3.71 x 10^5 and 3.15 x 10^5 ml^{-1} respectively. Mengesha (2013) identified the enzyme combinations of chitinase (5 mg/ml^{-1}), β-1,3-glucanase (3 $mg\ ml^{-1}$), driselase (10 $mg\ ml^{-1}$) and lyticase (5 $mg\ ml^{-1}$) prepared in 0.01 *M* phosphate buffer containing 0.6 *M* sodium chloride (NaCl), pH 6, for optimum protoplast yields of *P. florida* strains *viz* Pf5 and FRD2; and *V. volvacea* (Vv12).

5.19.3.3. Effect of Osmotic Stabilizers

Selection of osmotic stabiliser can significantly influence the rate of protoplast liberation and its suitability varies with species, owing to their differences in cell wall compositions (Cheng and Belanger, 2000). Among the evaluated osmotic stabilizers (0.6 *M* KCl and 0.6 *M* sucrose) prepared in 0.1 *M* phosphate buffer, pH 6.0 best results were obtained with 0.6 *M* KCl which produced 5.85 x 10^7 and 5.93 × 10^7 protoplasts ml^{-1} for *P. cystidiosus* (non-coremial) and *P. opuntiae* respectively. Similar to the present study, 0.6 *M* KCl was used as osmotic stabiliser for isolation of protoplasts from *P. pulmonarius* and *P. florida* (Eyini *et al.*, 2006), *Fusarium oxysporum* and *Fusarium pallidoroseum* (Naseema and Elizabeth, 2010) and *P. eous* and *P. flabellatus* (Parani and Eyini, 2010). Djajanegara and Masdukia (2010) identified 0.6 *M* $MgSO_4.7H_2O$ dissolved in 0.01 *M* phosphate buffer (pH 5.8) for optimum release of protoplasts from *P. florida* and *P. cystidiosus*.

5.19.3.4. Time of incubation

In the present study, a constant increase and subsequent decline in yield of protoplasts was observed with increasing time of incubation of *Pleurotus* spp. Five and four hours hydrolysis of 3 and 4 day old mycelium of *P. cystidiosus* (non-coremial) and *P. opuntiae* respectively was standardised for optimum release of protoplasts. The decrease in protoplast yield with an increased period of incubation was due to excessive exhaustion of protoplasts from the mycelia, leaving none in the mycelial debris. Also the potency of the enzyme solution to degrade the mycelia might get decreased, leaving some hyphae undigested

(Mengesha, 2013). Mallick and Sikdar (2015) recorded that, cell wall degrading enzymes released protoplasts from *P. florida* (1.5×10^7 protoplast g^{-1} tissue) and *L. squarrosulus* (9.6×10^6 protoplast g^{-1} tissue) after 12 and 10 h of incubation respectively.

Microscopical observation revealed the successive release of small nonvacuolated and large vacuolated protoplasts, reflecting differences in susceptibility of the different regions of the hyphae to the lytic enzymes, *i.e.* newly synthesized wall at the hyphal tip get more readily degraded than the wall in older regions. Also, the two types of protoplasts released may have differences in their potential to undergo regeneration as suggested by Zalokar (1959). The smaller protoplasts released from the terminal region have greater capacity for regeneration as this region of the hypha has the greater ability for wall synthesis, compared, to vacuolated protoplasts from the older parts.

5.19.4. Regeneration of protoplasts

Regeneration of protoplasts requires optimum hypertonic culture media where cell wall regeneration takes place. The optimum osmotic stabilizers supplemented into regeneration media also differed with different species. Protoplasts of *P. opuntiae* and *P. cystidiosus* (non-coremial) regenerated in to small colonies when malt yeast extract glucose medium (MYG) with 0.6 *M* KCl (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) was used as regeneration medium at 24 ^{0}C. Reversion of protoplasts was studied in solid regeneration media because wall components, their precursors, or enzymes involved in their synthesis, may get lost into the liquid medium whereas they can be retained in the solid medium (Lampen, 1968). Yanagi *et al.* (1985) identified sucrose as the best osmotic stabiliser for *A. bisporus* and *A. bitorquis*. Parani and Eyini (2010) recorded a regeneration efficiency of 0.28 and 0.24per cent for *P. eous* and *P. flabellatus*, respectively.

Regeneration of protoplasts resulted in the formation of a chain of yeast-like structure followed by the development of normal mycelium. Gabriel (1968) recommended the removal of snail enzymes before transferring protoplasts to the regeneration medium for regeneration. This was because, snail enzymes partially block the synthesis of the cell wall and the protoplasts transform into incessantly growing giant formations. Low regeneration values of protoplasts may be due to the clumping of contiguous protoplasts after centrifugation (Deed and Seviour 1989), high proteolytic activities of enzyme preparations, prolonged cell wall exposure, differences in the stability of protoplasts of varying phenotypes (Gold *et al.* 1983) and cultivation conditions (Kelkar *et al.*, 1990). Protoplasts that lack the ability to regenerate presumably either lack nuclei or were damaged at some point during or after the enzyme treatment (Wubie *et al.*, 2014).

5.19.5. Polyethylene glycol (PEG) mediated fusion and regeneration

Somatic hybridization through fusion of protoplast is a powerful and very useful parasexual technique for transformation/ engineering of higher plants and microbial strains, especially the fungi and actinomycetes. Standardising the concentration of PEG and osmotic stabilizers is essential to prevent shrinking, bursting and breaking of fused protoplasts (Lalithakumari, 2000). In the present study, fusion mixture comprised of PEG (molecular weight 4000, 40 per cent), calcium chloride (0.05 *M*) and glycine (0.05 *M*) using 0.6 *M* KCl was standardised for optimum protoplast fusion between *P. opuntiae* and *P. cystidiosus* (non-coremial); and recorded a regeneration frequency of 0.21 per cent. Parani and Eyini (2010) standardised PEG induced fusion experiments between *P. eous* and *P. flabellatus*, with a fusion frequency of 0.18 per cent. Selvakumar *et al.* (2015) standardised protoplast fusion between *P. ostreatus* var. *florida* and *P. djamor* var. *roseus.* They standardised 40 g PEG in 100 ml of 0.05 *M* calcium chloride dihydrate ($CaCl_2.2H_2O$) as optimum mixture for protoplast fusion.

5.19.6. Confirmation and evaluation of strains/hybrids

5.19.6.1. Selection on the Basis of Radial Growth

Gharehaghaji *et al.* (2007) suggested that mycelia of hybrids produced faster and thicker mycelial mat than those of parent cultures. This is one of the most important adaptive characteristic that determines suitability towards neo-physiological condition and high biological efficiency.

Eight fusant lines were obtained from the protoplast fusion experiment *viz.*, F1, F2, F3, F4, F5, F6, F7 and F8, of which F6 took the minimum time for completion of mycelial growth (4.01 days), showing white, thick, fluffy and concentric growth, with initiation of primordia, followed by F8 (4.33 days). Some putative hybrid fusants failed to grow in the subsequent subculturing processes due to unstable genotype or loss of genotype. Similar finding was observed by Mukherjee and Sengupta (1987), Chauhan (2014). Dhitaphichit and Pornsuriya (2005) obtained 412 regenerated colonies of which two were selected as fusants for posessing clamp connections on their mycelia, with significantly faster growth and larger size compared to the parental strains. PEG mediated protoplast fusion between *Pleurotus florida* and *Lentinus squarrosulus* successfully developed twelve somatic hybrid lines using double selection screening method (Mallick and Sikdar, 2015). Rosnina (2017) screened out two hybrid cultures *viz.*, P1XC9 and P3XC8 which exhibited thicker mycelium, high colony density and faster growth rate (8.5 mm day^{-1}, 8.2 mm day^{-1}) compared to *P. pulmonarius* and *P. citrinopileatus*. Contrary to the above findings, Gharehaghaji *et al.* (2007) concluded that fluffy type hybrid colonies should be avoided in the protoplast fusion between *P. ostreatus* strains.

5.19.6.2. Spawn production trials with the protoplast isolates and fusants

Comparative spawn production studies revealed that F6 (8.32 days) took the minimum time for mother spawn production followed by F8 (10.30 days) with

thicker and fluffy growth. Singh *et al.* (2007) recorded that fusants between *A. bisporus* and *Agaricus bitorquis* with a growth rate less than 1.4 mm day^{-1} failed during spawn running phase. They identified that, eleven isolates never completed spawn running process even after 40 d whereas, isolate H showed the least growth rate (0.55 mm day^{-1}). They screened out nine isolates which successfully completed the spawn running on non-composted substrate, with strandy, fluffier and good aerial growth, with or without sectoring. Kaur and Kapoor (2014) reported that, hybrid strain PFPS-131 completely impregnated the wheat straw in 19 days of incubation compared to parental strains *viz.*, *P. sajor-caju*-3 (35 days) and *P. florida*-5 (33 days), suggesting a very short cropping cycle and multiple crops in one season.

5.19.6.3. Cultivation trials with the protoplast isolates, fusants and second generation

In the present study, eight isolates were induced to form fruiting bodies, of which three isolates *viz.*, F4, F6 and F8 gave normal, distinct fruiting bodies. Morphological variations among the hybrid lines proved that the genome constitution of the independent hybrid lines was not equal and their differences in morphology resulted from gene recombination. Fruiting body of F4 was morphologically in between those of parents, whereas, F6 and F8 was similar to those of parents *viz.*, *P. cystidiosus* (non-coremial) and *P. opuntiae* respectively. F6 and F8 were stable in their morphological characters; and recorded improved biological efficiency over the parents in both generations tested (Fig 9). However, F4 was not stable and segregated in the second generation. Whether this is the result of the original cytoplasmic variation or of some procedure during the protoplast technique, such as the selection of vital cells, is unknown and needs further investigation (Magae *et al.*, 1985). They also developed six isolates from the dikaryotic regenerants which were able to form normal fruiting bodies, yielding 18 per cent more than the parent. Kaur and Kapoor (2014) developed somatic hybrids between *P. florida* and *P. sajor-caju viz.*, PFPS-47 and PFPS-101, which gave significantly higher yield (88.8 and 82.2 per cent respectively)

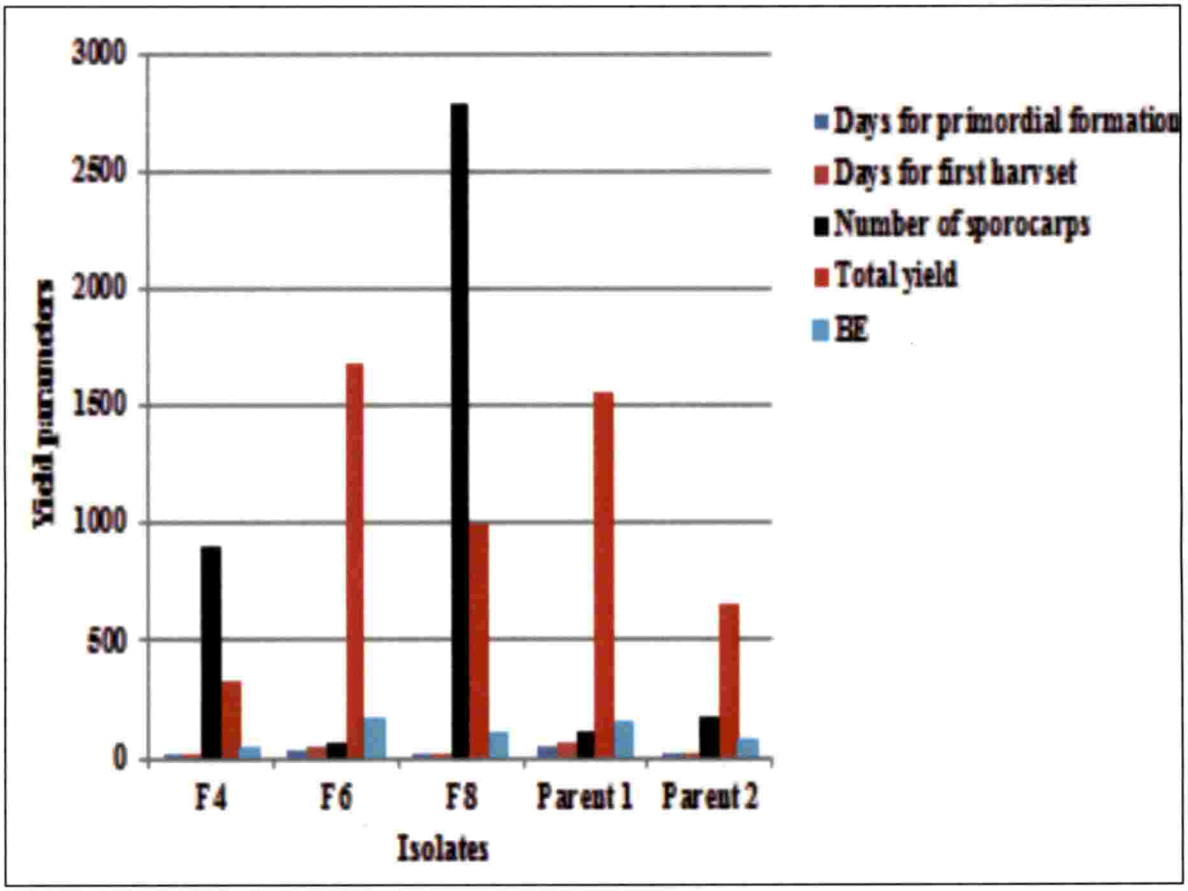


Fig 9. Comparative performance of protoplast isolates of parents and fusants (second generation) in rubber wood sawdust.

Parent 1: *P. cystidiosus* (non-coremial)

Parent 2: *P. opuntiae*

than the parent strains *viz.*, PSC-3 (34.5 per cent) and PF-5 (68.7 per cent). Selvakumar *et al.* (2015) obtained two hybrid strains through protoplast fusion between *P. ostreatus* var. *florida* and *P. djamor* var. *roseus*. The hybrid strains showed intermediate morphology in fruiting body characteristics to parents and recorded higher biological efficiency (138.97 per cent) than the parental strains (110.48-119.19 per cent). Rosnina (2017) recorded that, sporocarps of somatic hybrids between *P. pulmonarius* and *P. citrinopileatus viz.*, P8XC7 and P13XC7 were lung shaped similar; and was morphologically similar to *P. pulmonarius* with a little variation in colour *i.e.* pale grey colour at center of pileus. On the contrary, pileus of P19XC5 different from both the parental strains and produced fleshy and thicker sporophore with improved biological efficiency and durable shelf life. Sporocarps of the third generation belonging to P19XC5 showed consistency in morphological features *i.e.* crowded lamellae, bigger and thicker pileus with wavy edge, pleasant smell and less spores. The present experiment confirmed that, protoplast fusion is an appropriate method for creating interspecific hybrids in the genus *Pleurotus* as suggested by Dhitaphichit and Pornsuriya (2005). The somatic hybrids obtained through this study are not the end product and they could serve as resource material for further studies to give us insight about the basic genetics of basidiomycetes mating type genes, clamp formation, mode of sexuality and mushroom improvement programmes (Chakraborti and Sikdar, 2008).

Summary

6. SUMMARY

Survey for *P. cystidiosus* and *P. opuntiae* identified the coremial isolates of *P. cystidisous* in Vellayani from *Saracca indica* and *Eucalyptus globus* logs. Non-coremial isolates of *P. cystidiosus* were collected from Chirayinkeezhu, found growing on fallen wood logs of *Mangifera indica*. Another non-coremial isolate was developed from dowel spawns procured from Germany. Isolates of *P. opuntiae* from Vellayani was recorded from *Mangifera indica* L logs, *Cocos nucifera* L. and *Areca catechu* L. logs.

The morphological studies of *Pleurotus* spp. revealed that coremial and non-coremial isolates of *P. cystidiosus* were light brown to creamish white, oyster shaped, smooth surfaced with fringed margin and pileus size ranging from 18.33 cm x 15.4 cm to 19.70 cm x 16.51 cm and 15 cm x 11.52 cm, respectively. Sporocarps of *P. opuntiae* were white to creamish white in colour, oyster shaped, smooth surfaced with fringed margin and pileus of size 5.10 cm x 4.02 cm to 9.80 cm x 5.35 cm. Spore prints of the *Pleurotus* spp. isolates were white in colour. Hyphae of the *Pleurotus* spp. were septate, branched, hyaline, aerial as well as submerged with distinct clamp connection. Basidiospores of *Pleurotus* spp. were hyaline, oval, sub cylindrical to cylindrical shape. The basidiospores stained dark blue and red in one per cent lactophenol cotton blue and one per cent congo red respectively. Cystidial studies revealed the presence of cheilocystidia and pleurocystidia for *P. cystidiosus* (non-coremial), *P. cystidiosus* (coremial) and *P. opuntiae*.

Three isolates of *P. opuntiae viz.*, PO1 and PO2, two coremial isolates of *P. cystidiosus viz.*, PC1 and PC2; and two non-coremial isolates of *P. cystidiosus viz.*, PNC1 and PNC2 were obtained through isolation by tissue culturing and pure culturing by hyphal tip method. Mycelium of PC1 and PC2 isolates showed small protuberances on the entire tissue simultaneously followed by the development of

little white stalk with tiny black watery droplets. The isolates screened for further studies were PC2, PNC1 and PO1; and were molecularly characterised by ITS sequencing and accession numbers *viz.*, KY214254, KY887023, KY214255, KY214256 and KY214257 respectively were retrieved from NCBI GenBank.

The screened isolates of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* recorded the maximum growth on PDPA medium amended with yeast one per cent under dark condition. The optimum temperature for the growth was 30 ^{0}C, 25 to 30 ^{0}C and 25 ^{0}C, respectively, whereas optimum pH was 8, 8 and 7 to 8, respectively, based on the minimum time taken for complete coverage of petri dish and thickness of mycelial growth. The submerged culture production of isolates revealed that PDPB recorded the highest fresh weight and biomass for the three isolates.

P. cystidiosus (coremial) was characterised by the specialised feature named coremia, representing its anamorphic stage-*Antromycopsis macrocarpa*. Microscopical observation of the aerial hyphae showed stalk-like cells whose tops were fitted with black liquid droplets, containing round and elliptical conidial spores. Melanin pigment from coremial liquid of *P. cystidiosus* was extracted and characterised by bio-chemical tests and UV spectra. UV spectrum showed a characteristic absorption peak around 250 nm. Also the extracted melanin showed efficient free radical scavenging activity of DPPH radical.

Best substrate and amendment for *P. cystidiosus* (coremial) and *P. opuntiae* was identified as sorghum with yeast one per cent whereas, paddy grains with yeast one per cent was the best for *P. cystidiosus* (non-coremial). However, spawns prepared from rubber wood sawdust amended with yeast one per cent, recorded the maximum shelf life for *P. cystidiosus* (non-coremial), *P. cystidiosus* (coremial) and *P. opuntiae*. Screening of substrates and amendments revealed

that, spraying of rubber wood sawdust with 1 *M* KH_2PO_4 2.5 per cent was the best combination for *P. cystidiosus* (non-coremial), whereas rubber wood sawdust amended with either wheat bran or neem cake 4 per cent gave the maximum BE for *P. opuntiae*.

Multilocational trials revealed that *P. cystidiosus* (coremial) failed to produce significant fruiting bodies either in Trivandrum or Idukki. The study screened out *P. cystidiosus* (non-coremial) and *P. opuntiae* as the best isolates, suited for the climatic conditions of Idukki and Trivandrum respectively.

Screening of different amendments revealed that, wheat bran 4 per cent was the best amendment for *P. cystidiosus* (non-coremial) in paddy straw and coirpith with BE of 125.52 and 37.57 per cent, respectively, whereas spraying of rubber wood sawdust with 1 *M* KH_2PO_4 2.5 per cent gave the maximum BE (192.76 per cent). In case of *P. opuntiae*, wheat bran 4 per cent was the best amendment in paddy straw and coirpith (BE of 80.60 and 46.03 per cent respectively), whereas the best amendments in rubber wood sawdust were either wheat bran 4 per cent or neem cake 4 per cent (BE of 91.37 and 91.38 per cent respectively).

Sporocarps of *P. cystidiosus* (coremial) and *P. cystidiosus* (non-coremial) took an average of five days from the day of pinhead formation to complete maturity, whereas, sporocarps of *P. opuntiae* took an average of four days.

Comparative spawn production studies revealed that minimum time for spawn run was recorded with *P. cystidiosus* (non-coremial) in paddy grains (amended with yeast 1 per cent) (5.61 days) and *P. opuntiae* in sorghum grains (amended with yeast 1 per cent) (9.28 days). Comparative mushroom production

studies revealed that cultivation of *P. cystidiosus* (non-coremial) on rubber wood sawdust sprayed with 1*M* KH_2PO_4 2.5 per cent recorded maximum crop period (145.30 days), weight of sporocarps (35.80 g), number of sporocarps (106.90), total yield from three harvest (3858.60 g) and BE (192.2 per cent) whereas, *P. opuntiae* in rubber wood sawdust amended with wheat bran 4 per cent gave the maximum number of sporocarps (137.60 days), total yield from three harvest (913.60 g) and BE (91.36 per cent).

Nutritional studies revealed that *P. cystidiosus* (non-coremial) recorded the maximum moisture (94.05 per cent), starch (200.55 mg g^{-1}), protein (30.20 mg g^{-1}), fat (4.25 per cent), antioxidants (485.45 µg Eq.g^{-1} of ascorbic acid)), beta carotene (25.69 µg 100 mg^{-1}), polyphenol (7.55 mg g^{-1}) and energy (359.45 Kcal) values. MTT assay revealed that, dried extracts of *P. cystidiosus* (non-coremial) and *P. opuntiae* at concentration of 100 µg ml^{-1} recorded the highest anti-proliferative effect on colon cancer cells, with minimum cell viability of 45.99 and 29.27 per cent, respectively. Direct microscopic observations revealed indications of cytotoxicity, with LD-50 values of 78.29 and 55.04 µg ml^{-1} respectively.

Harvested mushrooms of *P. opuntiae*, *P. florida, P. cystidiosus* (non-coremial) and *P. eous* recorded shelf life of 24, 24, 48 and 24 h respectively in natural condition whereas, 3, 3, 5 and 3 days were recorded when preserved in refrigerated condition. Assessment of cooking quality and sensory evaluation revealed that, masala curry prepared from *P. cystidiosus* (non-coremial) recorded the maximum values for appearance (9.1), colour (9.3), flavor (9.0), texture (9.0), taste (9.2), aroma (9.1) and overall acceptability (9.4) among all other recipes of *Pleurotus* spp. studied. Soaking of sporocarps in CA treatment (1 per cent) for 15 min, followed by mechanical drying was identified as the best post harvest treatment for *P. cystidiosus* (non-coremial) and *P. opuntiae*.

Compatibility studies revealed the occurrence of barrage reaction between the crosses *viz*., *P. cystidiosus* (coremial) x *P. opuntiae* and *P. cystidiosus* (non-coremial) x *P. opuntiae.* Dual biochemical markers *viz*., carbendazim (1 mM) and vanillin (0.05 per cent) were selected for screening out the fusants between *P. cystidiosus* (non-coremial) and *P. opuntiae*. Three days old *P. cystidiosus* (non-coremial) and four days old *P. opuntiae* recorded the maximum protoplast yield at 5 and 4 h after incubation respectively with 0.6 *M* KCl and 30 mg ml^{-1} of enzyme consortium.

Protoplasts of *P. cystidiosus* (non-coremial) and *P. opuntiae* regenerated in to small colonies when malt yeast extract glucose medium (MYG) with 0.6 *M* KCl (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) was used as regeneration medium, at 24 ^{0}C. Fusion mixture comprised of PEG (molecular weight 4000, 40 per cent), calcium chloride (0.05 *M*) and glycine (0.05 *M*) using 0.6 *M* KCl was standardised for optimum protoplast fusion between *P. cystidiosus* (non-coremial) and *P. opuntiae*; and recorded a regeneration frequency of 0.21 per cent.

Eight fusant lines were obtained from protoplast fusion *viz*., F1, F2, F3, F4, F5, F6, F7 and F8, of which F6 took the minimum time for completion of mycelial growth (4.01 days), showing white, thick, fluffy and concentric growth, with initiation of primordia, followed by F8 (4.33 days). Among the fusants, F6 (8.32 days) took the minimum time for mother spawn production followed by F8 (10.30 days), with thicker and fluffy growth. The eight isolates were induced to form fruiting bodies of which three isolates *viz*., F4, F6 and F8 gave normal, distinct fruiting bodies.

Fruiting body of F4 was morphologically in between those of parents, whereas, F6 and F8 was similar to those of parents *viz.*, *P. cystidiosus* (non-coremial) and *P. opuntiae*, respectively. F6 and F8 were stable in their morphological characters; and recorded improved biological efficiency over the parents in both generations tested. However, F4 was not stable and segregated in the second generation.

The present study revealed that, *P. cystidiosus* (non-coremial) is the best oyster mushroom species suited for low temperature areas like Idukki, Munnar and Wayanad whereas, *P. opuntiae* is recomended for high temperature regions. The two newly identified species can be cultivated successfully in Kerala by utilizing the locally available materials. The attractive colour, oyster shape, taste, nutritional as well as medicinal attributes enhances the chances of easy acceptance and popularity among the farmers. Standardization of PEG mediated protoplast fusion between *P. cystidiosus* (non-coremial) and *P. opuntiae* screened out two fusant lines (F6 and F8) with higher BE which can be used for future breeding programmes.

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STRAIN IMPROVEMENT OF OYSTER MUSHROOMS– *PLEUROTUS CYSTIDIOSUS* O.K.MILL AND *PLEUROTUS OPUNTIAE* (DURIEU AND LEV.) SACC.

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(2015 - 21 - 008)

ABSTRACT

of the thesis submitted in partial fulfilment of the requirements for the degree of

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ABSTRACT

The present study entitled "Strain improvement of oyster mushrooms: *Pleurotus cystidiosus* O.K.Mill and *Pleurotus opuntiae* (Durieu and Lev.) Sacc." was carried out in College of Agriculture, Vellayani during 2015-2018, with the objective to standardize the techniques for production of oyster mushrooms: *P. cystidiosus* and *P. opuntiae*; and to study their morphological, physiological and cultural characteristics as well as nutritional and organoleptic qualities; and to undertake genetic improvement by protoplast fusion.

The mushrooms were collected from two locations of Thiruvananthapuram and three fast growing isolates of *Pleurotus* spp. *viz.*, PC2 (Vellayani), PNC1 (Chirayinkeezhu) and PO1 (Vellayani) were selected for the study. These isolates were identified as *P. cystidiosus* subsp. *abalonus*, *P. cystidiosus* and *P. opuntiae* using internal transcribed spacer (ITS) primers and subsequent sequencing; and registered at Genbank database with accession numbers KY214254, KY887023 and KY214255 respectively.

The fast growing isolates of *P. cystidiosus* (coremial), *P. cystidiosus* (non-coremial) and *P. opuntiae* recorded maximum growth on PDPA amended with one per cent yeast under dark condition. The optimum temperatures for the growth were 30 ^{0}C, 25 to 30 ^{0}C and 25 ^{0}C respectively whereas, the optimum pH were 8, 8 and 7 to 8 respectively.

Studies with different substrates and amendments for spawn production revealed that sorghum with one per cent yeast was the best for *P. cystidiosus* (coremial) and *P. opuntiae* whereas, paddy grains with one per cent yeast for *P. cystidiosus* (non-coremial). Experiments with different substrates and amendments for mushroom production revealed that rubber wood sawdust

sprayed with 2.5 per cent of 1 *M* potassium dihydrogen phosphate recorded the maximum BE for *P. cystidiosus* (non-coremial) (192.76 per cent). *P. opuntiae* recorded the maximum BE in rubber wood sawdust amended either with 4 per cent neem cake (91.38 per cent) or wheat bran (91.37 per cent). Major insect pests observed in the beds of *Pleurotus* spp. were phorid flies, spring tails, black ants and staphylinid beetles. The competitor moulds observed were different species of *Coprinus*, *Aspergillus*, *Penicillium* and *Trichoderma*.

Sporocarps soaked in one per cent CA for 15 minutes followed by mechanical drying and powdering was the best post harvest treatment for both *P. cystidiosus* (non-coremial) and *P. opuntiae*. Mycelium of *P. cystidiosus* (coremial) showed black coremial structures, representing its asexual stage (*Antromycopsis broussonetiae* Pat. & Trab.). The coremia comprised of elliptical (16.31 µm x 7.48 µm) and round conidia (8.06 to 8.49 µm). The black colour of coremia was due to melanin which was extracted (255.56 mg l^{-1}) and characterized. The performance of long duration *P. cystidiosus* (non-coremial) and short duration *P. opuntiae* was compared with two ruling mushrooms of Kerala *viz*., long duration *P. florida* (Mont.) Singer and short duration *P. eous* (Berk.) Sacc. The study revealed that *P. cystidiosus* (non-coremial) and *P. opuntiae* showed higher BE compared to *P. florida* and *P. eous,* respectively. *P. cystidiosus* (non-coremial) recorded maximum moisture (94.05 per cent), starch (200.55 mg g^{-1}), protein (30.2 mg g^{-1}), fat (4.25 per cent), antioxidants (485.45 µg equivalent gram of ascorbic $acid^{-1}$), beta-carotene (25.69 µg 100 mg^{-1}), polyphenols (7.55 mg g^{-1}) and energy (359.45 Kcal) compared to other *Pleurotus* spp. Sensory evaluation of mushroom products made from the species of *Pleurotus* was done and masala curry prepared from *P. cystidiosus* (non-coremial) scored the maximum value for overall acceptability. Shelf life of *P. cystidiosus* (non-coremial) was higher (5 days) compared to *P. opuntiae, P. florida* and *P. eous* (3 days each) in perforated poly propylene covers stored under refrigeration.

Vanillin (0.05 per cent) and carbendazim (1 m*M*) were selected as dual biochemical markers for the PEG mediated protoplast fusion. Three days old *P. cystidiosus* (non-coremial) and four days old *P. opuntiae* recorded the maximum protoplast yield at five and four hours after incubation respectively with 0.6 *M* KCl and 30 mg ml^{-1} of enzyme consortium. Eight fusant lines with varied mycelial characters were obtained. Among fusants, F6 and F8 did not segregate in the second generation whereas, F4 segregated. F6 and F8 recorded higher BE of 168.05 and 99.95 per cent respectively compared to the parental lines and other fusants. Sporocarp of F6 and F8 was morphologically similar to *P. cystidiosus* (non-coremial) and *P. opuntiae* respectively; and F8 also exhibited low temperature adaptability.

The present investigation indicated the exploitability of two promising isolates *viz. P. opuntiae* for tropical areas and *P. cystidiosus* (non-coremial) for cooler regions of Kerala using locally available materials and the standardized cultivation practices. The present study also standardized the protoplast fusion technique between *P. cystidiosus* (non-coremial) and *P. opuntiae*; and two fusant lines (F6 and F8) recorded higher BE which can be used for future breeding programmes.

APPENDIX – I

DATASHEET

Date of collection.....

Collected by..... Locality.....

GENERAL

Substrate :

Habitat : Terrestrial / Lignicolous / Epixylose / Coprophilous

Habit : Solitary / Scattered / Gregarious

Pileus

Shape : Convex/Infundibuliform/Umbonate/Petaloid/Flabelliform/Depressed

Colour :

Size : Diameter

Thickness

Texture : Soft/ Brittle/ Fleshy/ Fragile/ Coriaceous/ Membraneous

---

Stipe

Shape : Clavate

Size : Length :

Diameter :

Attachment to pileus : Lateral/ Eccentric/ Central/ Resupinate

Surface : Glabrous/Scaly/Smooth/Pubescent/Fibrillose

Basal part	: Globular/Bulbous/Fusoid/Cylindrical

APPENDIX – I (Continued)

Gills	
Arrangement	: Remote/Free/Decurrent/Adnate/Adnexed/Sinuate
Texture	: Soft/Brittle/Waxy/Thick/Papery/Opaque
Margin	: Smooth/Wavy/Serrate/Fimbriate/Dentate
Size	: Number per cm
Veil	
Type	: Present/ Absent
Annulus	
Type	: Present/ Absent
Volva	
Type	: Present/ Absent
Spore print	
Colour	:
Spores	
Colour	:
Shape	: Ovate/Elliptical/Globose/Epiculate/Cylindrical/Fusiform/ Angular/Echinulate/Recticulate/Ovoid/Pyriform
Reaction with	
Cotton blue	: Cyanophilic/ Acyanophilic
Melzer's reagent	: Amyloid/ Dextrinoid/ Nonamyloid

APPENDIX – II

STAIN FOR PREPARATION OF MICROSCOPIC SLIDES

1. Lactophenol cotton blue (100 ml)

Phenol	-20 g
Lactic acid	-20 ml
Glycerol	-40 ml
Cotton blue	-0.05 g

APPENDIX III

BUFFERS FOR PCR ANALYSIS

1. 50 x TAE buffer (Tris –Acetate-EDTA) (pH 8.0)

Tris base	-242.0 g
Acetic acid	-57.1 ml
0.5 *M* EDTA	-100 ml

Add distilled water to a final volume of 1 litre.

2. Sample loading buffer (6x)

0.25 per cent Bromophenol blue

40 per cent (w/v) sucrose in water

APPENDIX IV

GENE SEQUENCE OF THE ISOLATES UNDER STUDY

>KY214255

AAGGATCATTAATGAATTACAAAAGCTTTTGAAGTTGTTTTGCTGGTCT
CTAGGGACATTGTGCACGCTTCATTAGTTTCCACTTCATACCCCTGTGC
ACCTTTGATAGATTTCGGTTTGGGTTATCCTTTGGTTTTTTTTCTTAATT
GAAAGGCCTTTGGTTTCCTTAAAACGACTTCTATACTATACCACACACC
AAATGTATGTTTTATAATGAATGGTTTATAATGACAAGGCCATGAGCC
TTATAAACTTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCG
AAGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCTATGACTTT
ATTGTTGTAGCTGTTTGGATTGTTGGGGGTTGCTGGCTTCTTTCTTTGA
AGTCGGCTCCTCTTAAATGCATTAGCGGGACTTTGTTGCCTCTGCGCAT
AGTGTGATAATTATCTACGCTAGACGCATGCAATTCTTATATTGTCCAG
CTTTCTAATCGTCTCAAGGGACAATTACTTTGACAATTTGACCTCAAAT
CAGGTAGGACTACCCGCTGAACTTAA

> KY214254

AAGGATCATTAATGAATTCACTCATGAAGCTGATGCTGGTCTCTCGGG
ACATGTGCACGCTTCATAAGTACATTCAACCACTTGTGCACTTTTGATA
GATTCGCAGAGTTGCCCTCTCAGGTCAGTAAATGACTTGGTTGGTCGG
GATTGTCACAGTCCTGGCTTTGACTTTGTGGGTCTATTATCTTATACAC
ACTTGTATGTCCATGAATGTTATTTTCTTGGGCCATGTGCCTATAAAAC
CTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGC
ATGCCTGTTTGAGTGTCATTAAATTCTCAAATCTATAGAGCTTTTTTGT
GATATAGATTTGGATTGTTGGGGGCTGCTGGCTTTTTACCAAGTTGGCT
CCTCTTAAATGCATTAGCGGGACTTTATTGCCTCTGCGCACAGTGTGAT

AATTATCTACGCTGGCCGACATGCAATGACTTTACAAGTCCAGCTTTCT
AACTGTCTTTCAAGACAATGACTTGACAATTTGACCTCAAATCAGGTA
GGACTACCCGCTGAACTTAA

> KY887023

GTTGCTGGCCTCTAGGGGCATGTGCACGCTTCACTAGTCTTTCAACCAC
CTGTGAACTTTTGATAGATCTGTGAAGTCGTCTTTCAAGTCGTCAGACT
TGGTTTGCTGGGATTTAAACGTCTCGGTGTGACAACGCAGTCTATTTAC
TTAACACACCCCAAATGTATGTCTACGAATGTCATTTAATGGGCCTTGT
GCCTATAAACCATAATACAACTTTCAACAACGGATCTCTTGGCTCTCG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTAT
TCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCACTTT
GGTTTTTTTCCAATTGTGATGTTTGGATTGTTGGGGGCTGCTGGCCTTG
ACAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTGCCTCTG
CGCATGATGTGATAATTATCACTCATCAATAGCACGCATGAATAGAGT
CCAGCTCTCTAATCGTCCGCAAGGACAATTTGACAATT

> KY214257

AAGGATCATTAATGAATTCACTATGGAGTTGTTGCTGGCCTCTAGGGG
CATGTGCACGCTTCACTAGTCTTTCAACCACCTGTGAACTTTTGATAGA
TCTGTGAAGTCGTCTCTCAAGTCGTCAGACTTGGTTGCTGGGATTTAAA
CGTCTCGGTGTGACTACGCAGTCTATTTACTTACACACCCCAAATGTAT
GTCTACGAATGTCATTTAATGGGCCTTGTGCCTTTAAACCATAATACAA
CTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTTG
AGTGTCATTAAATTCTCAAACTCACTTTGGTTTCTTTCCAATTGTGATG
TTTGGATTGTTGGGGGCTGCTGGCCTTGACAGGTCGGCTCCTCTTAAAT
GCATTAGCAGGACTTCTCATTGCCTCTGCGCATGATGTGATAATTATCA
CTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAATCGTCCGCA

AGGACAATTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGA
ACTTAA

> KY214256

TAATGAATTACAAAAGCTTTTGAAGTTGTTTTGCTGGTCTCTAGGGACA
TTGTGCACGCTTCATTAGTTTCCACTTCATACCCCTGTGCACCTTTGAT
AGATTTCGGTTTGGGTTATCCTTTGGTTTTTTTTCCTAAATTGAAAGGC
CTTTGGTTTCCTTAAAACGACTTCTATACTATACCACACACCAAATGTA
TGTTTTATAATGAATGGTTTATAATGACAAGGCCATGAGCCTTATAAA
CTTAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAA
GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAAGGGC
ATGCCTGTTTGAGTGTCATTAAATTCTCAAACTCTATGACTTTATTGTT
GTAGCTGTTTGGATTGTTGGGGGTTGCTGGCTTCTTTCTTTGAAGTCGG
CTCCTCTTAAATGCATTAGCGGGACTTTGTTGCCTCTGCGCATAGTGTG
ATAATTATCTACGCTAGACGCATGCAATTCTTATAGTTGTCCAGCTTTC
TAATCGTCTCAAGGGACAATTACTTTGACAATTTGACCTCAAA

APPENDIX V

COMPOSITION OF DIFFERENT MEDIA

a) Potato Dextrose Agar (PDA)

Potato : 200 g

Dextrose : 20 g

Agar-agar : 20 g

Distilled water : 1 litre

b) Malt Extract Agar (MEA)

Malt extract : 25 g

Agar-agar : 20 g

Distilled water : 1 litre

c) Oat Meal Agar (OMA)

Oat flakes : 30 g

Agar-agar : 20 g

Distilled water : 1 litre

d) Carrot Extract Agar (CEA)

Grated carrot : 20 g

Agar-agar : 20 g

Distilled water : 1 litre

e) Potato Dextrose Peptone Agar (PDPA)

Potato : 200 g

Dextrose : 20 g

Agar-agar : 20 g

Peptone: 10 g

Distilled water : 1 litre

APPENDIX VI

CHARACTERISATION OF THE EXTRACTED MELANIN

a. Ammoniacal silver nitrate solution

5 ml of silver nitrate solution was pipetted in a conical flask with constant shaking or swirling. To the solution, concentrated ammonium hydroxide was added, drop by drop, until the precipitate formed was completely dissolved. 5 ml of sodium hydroxide was added and black precipitate was formed. Concentrated ammonium hydroxide was added drop by drop, with continuous swirling, until the precipitate dissolved. Silver nitrate solution was added drop by drop, until the solution became permanently cloudy. The resulting solution was diluted to 50 ml with distilled water. The solution should be used fresh.

b. 0.1 *M* Borate buffer (pH 8.8)

Stock solutions

A: 0.2 *M* solution Boric acid (12.4 g in 1000 ml)

B: 0.05 *M* solution of Borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml.

APPENDIX VII
BUFFER FOR MTT ASSAY

Phosphate buffered saline (PBS-pH 7.4)

Sodium chloride	-8.0 g
Potassium dihydrogen phosphate	-0.2 g
Disodium hydrogen phosphate	-1.1 g
Potassium chloride	-0.2 g
Sodium azide	-0.2 g
Water	-1000 ml

APPENDIX VIII

MUSHROOM RECIPES

a.Sauted mushroom

Ingredients

100 g Mushroom

10 g Big onion

5 g Green chilli

20 g Tomato

1 tsp. Coconut oil

1/8 tsp. Pepper powder

Turmeric powder, a pinch

Red chilli powder, a pinch

Salt to taste

Curry leaves, one spring

Method

Big onion, green chilli and tomato was sauted in hot oil. To the sauted mixture, mushroom pieces, salt and other ingredients were added. The mixture was again sauted and cooked by covering the vessel.

b.Mushroom masala curry

Ingredients

250 g Mushroom

10 g Big onion

5 g Green chilli

20 g Tomato

2 tsp. Coconut oil

1 tsp. Ginger-garlic paste

1/8 tsp. Pepper powder

¼ tsp. Cumin powder

¼ tsp. Cardamom powder

¾ tsp. Garam masala

¾ tsp. Red chilli powder

Turmeric powder, a pinch

Salt to taste

Curry leaves one spring

Method

Mushroom pieces and dry spices were separately sauted in coconut oil and kept aside. Green chilli, big onion, ginger-garlic paste, tomato and turmeric were fried till raw smell disappeared. To this mixture, water was poured and brought to boil. Mushrooms were then added to the mixture, half cooked, followed by the addition of garam masala, red chilli powder and turmeric powder. The mixture was again sauted and cooked by covering the vessel.

c. Mushroom soup

2 tablespoons butter

200 g sliced fresh mushrooms

1/4 cup chopped onion

6 tablespoons all-purpose flour

1/2 teaspoon salt

1/8 teaspoon pepper

1 cup butter

Method

Mushrooms and onion were sauted using butter over medium-high heat, until they turn tender. Flour, salt and pepper were mixed to form smooth broth and stirred with the prepared mushroom mixture. The mixture was brought to boil; cooked and stirred for 2 min, until it got thickened. Then heat was reduced and left for 15 min with occasional stirring, until flavours were blended.

d.Mushroom bhaji

200 g chopped mushrooms

1 tbsp. All purpose flour

I tbsp. Corn flour

1 and ½ tbsp. Rice flour

¼ tsp. Red chilli powder

1/8 tsp. Turmeric powder

½ tsp Garam Masala

¼ Salt (adjust per taste)

All the ingredients except oil and mushrooms were mixed, by adding water little by little to make a medium thick batter. The chopped mushroom were then added, covered with the batter and kept for 5 mins. The battered mushrooms were the fried in hot oil to get crispy bhajis.

APPENDIX IX

Hedonic rating scale for the evaluation of mushroom recipe

Particulars	Score
Like Extremely	9
Like Very Much	8
Like Moderately	7
Like Slightly	6
Neither Like nor Dislike	5
Dislike Slightly	4
Dislike moderately	3
Dislike Very Much	2
Dislike Extremely	1

APPENDIX X
MEDIA FOR TOTAL PLATE COUNT (TPC)

1. Martins Rose Bengal Agar medium

Glucose	-10 g
Peptone	-5 g
Potassium hydrogen phosphate	-0.5 g
Potassium dihydrogen phosphate	-0.5 g
Magnesium sulphate hydrate	-0.5 g
Rose Bengal	-30 mg
Agar	-12 g
Streptomycin sulphate	-30 mg

All ingredients are mixed (except streptomycin sulphate) and autoclaved. The media was cooled to 50^0C and streptomycin sulphate was added.

2. Nutrient agar medium (pH 7.4)

Peptone	-5 g
Beef extract	-1.5 g
Sodium chloride	-5 g
Agar	-15 g
Distilled water	-1000 ml

APPENDIX XI

BUFFER/MEDIUM FOR PROTOPLAST ISOLATION

1. 0.1 *M* Sodium phosphate buffer (pH 6.5)

Stock solutions

A: 0.2 *M* Solution of monobasic sodium phosphate (27.8 g in 1000 ml)

B: 0.2 *M* Solution of dibasic sodium phosphate (53.56 g $Na_2HPO_4.7H_2O$ in 1000 ml)

87.7 ml of A is mixed with 12.3 ml of B and diluted to 200 ml.

2. Malt yeast glucose medium (MYG medium, pH 6)

Malt extract	-20 g
Yeast extract	-20 g
Glucose	-20 g
Agar	-30 g
Water	-1000 ml

0.6 *M* potassium chloride (prepared in 0.01 *M* sodium phosphate buffer pH 6.5) was added to MYG medium, as an osmotic stabilizer.