STRAIN IMPROVEMENT AND PRODUCTION TECHNOLOGY OF MILKY MUSHROOM (*Calocybe indica* P. &C.)

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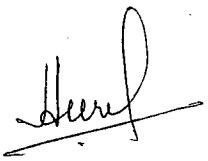
Dedicated to

My Everloving Amma

K.K.Sumam (Late)

DECLARATION

I hereby declare that this thesis entitled "**Strain improvement and production technology of milky mushroom (*Calocybe indica* P. &C.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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LIST OF ABBREVIATIONS

%	Per cent
γ	Gamma
°C	Degree Celsius
µg	Microgram
µl	Micro litre
BE	Biological efficiency
cm	Centimetre
et al.	And others
g	Gram
hrs	Hours
KR	Kilo rads
L-DOPA	L-3,4-dihydroxy phenyl alanine
M	Molar
mg	Milligram
min	Minute(s)
mM	Milli molar
PAL	Phenyl alanine ammonialyase
PDA	Potato dextrose agar
PO	Peroxidase
PPO	Polyphenol oxidase
RAPD	Random amplified polymorphic DNA
SMS	Spent mushroom substrate
TEMED	Tetramethyl ethylene diamine
TSS	Total soluble solids
UV	Ultraviolet

Introduction

1. INTRODUCTION

Mushrooms without leaves, without buds, without flowers yet they form fruit as food as a tonic and as medicine, the entire creation of nature is precious. Mushrooms have attracted the attention of man from very ancient times and the use of mushroom as food is as old as human civilization. In ancient religious writings like Vedas and Bible, there are references on the occurrence of mushrooms and their utilization as food as well as medicine. Mushroom cultivation is coming up as one of the most profitable and environment friendly enterprise among various horticultural crops. The cultivation of mushrooms dated back in 17^{th} century in France. The cultivation of mushrooms in its primitive forms must have been an ancient art. The abundance of agrowastes on the tropical countries possessed threat to environment. The agrowaste consisting of cellulosic and lignolytic materials are resistant to biodegradation and ruminant digestibility. Natural plant wastes, the lignocellulosic, are the basic substrates for growth and yield of mushrooms. Mushroom species represent efficient microbes gifted with unique ability to degrade lignin beside cellulose and hemicellulose and produce fruiting bodies. Other redeeming features of mushroom growing are that it requires limited space, needs no sunlight and fertile soil.

The diet of an average Indian comprises of calorie rich food stuffs but often highly deficient in proteins and ill effects of malnutrition are quite common in our country. The cultivation of edible mushrooms also helps in supplementing and complementing the nutritionally deficient cereals and are regarded as the highest producer of protein per unit of time, which helps in ameliorating the protein gap prevalent in our country. It is recommended by FAO as major protein source in developing countries. The mushrooms are hence referred to as "vegetarian meat". The mushrooms are good source of protein, minerals like sodium and potassium, essential amino acids like

lysine, tryptophan and vitamins, vitamin B complex, vitamin C and folic acid apart from having a pleasant flavour and taste.

Among the 2000 species of edible mushrooms, widely commercialized ones include *Agaricus*, *Pleurotus*, and *Volvariella*. The technique for the mushrooms can be primitive as in *Pleurotus* and *Volvariella* and highly industrialized as in *Agaricus*. Presently the world production of mushrooms is 55 lakhs tones which is not sufficient to meet the growing demands for culinary purpose with the existing cultivars alone. Ninety per cent of mushroom production is contributed by *Agaricus* sp. alone, which is highly temperature sensitive. In tropical regions where *Agaricus* is not suited, diversified cultivation involving different species suited to specification is very essential. India is blessed with varied agroclimates which makes it suitable for cultivation of different types of mushroom.

Calocybe indica an indigenous popular wild edible mushroom is a new entrant among the commercial domesticated ones. *Calocybe indica* popularly known as milky mushroom or summer mushroom due to its robust milky white large sporophores and delicious flavour. This mushroom was reported in India by Purkayastha and Chandra in 1974. First attempt on the induction of fruit bodies of *Calocybe indica* was made almost two decades ago. *Calocybe indica* normally occurs in the humus soil occurring in road side trees and forest trees. *Calocybe* has a biological efficiency of 60-70 per cent when grown under optimum conditions. The warm temperature requirement of the mushroom makes it suitable for relay cropping.

When compared with other mushrooms *Calocybe* spp. have a nutritive value of 17.39 per cent protein, 8.1 per cent carbohydrate, 0.92 per cent fat and 17 per cent fibre. It is also considered as a medicinal mushroom, as it prevents constipation due to high fibre content.

Usually mushrooms have very short shelf life and cannot be stored fresh for more than few days even under best storage conditions. But

Calocybe has a long shelf life even under normal conditions so offers wide export potential. The robust nature, milky white colour, delicacy, long shelf life, tropical nature and lucrative value have attracted the attention of both mushroom consumers and prospective growers. It is mainly cultivated in Tamil Nadu in South India. Though it is a mushroom with wide potential, yet it remains unexploited.

Lower yield compared to *Pleurotus*, process of casing and lack of marketing facilities hinder the production and acceptability of this mushroom in a commercial scale, even with its long shelf life.

The economy of Kerala is mainly based on agriculture. The typical homestead in Kerala is really a unique unit of mixed farming involving crops, cattle, poultry etc. In Kerala, we are now at a stage in which further expansion of area under cultivation of crops is almost impossible, as all the arable areas have been brought under cultivation. More intensive cultivation is attempted for increased yield and providing employment opportunities all the year round. The introduction of mushroom cultivation in our farming system is gaining importance in this context. The agricultural wastes which are very much in plenty in Kerala can be utilized for the production of mushrooms which are highly nutritious food materials.

The present study was conducted with the major objectives of obtaining elite strains of *Calocybe* from native flora followed by improvement in the production technology utilizing indigenous substrates, and development of strains of *Calocybe* suited to Kerala conditions. Attempts were made on the strain improvement of existing strains with desirable characters or combining characters using UV and gamma irradiation and hybridisation of isolates. The molecular characterization studies of hybrids and parents using RAPD were conducted to determine the genomic level expressions.

Review of Literature

2. REVIEW OF LITERATURE

2.1 SURVEY AND COLLECTION OF MUSHROOM MICROFLORA

Calocybe indica / Milky mushroom was first reported from India by Purkayastha and Chandra in 1974. Suharban (1987) conducted survey in different parts of Thiruvananthapuram for the monographic studies on the genus *Pleurotus*. Sarkar *et al.* (1988) reported the occurrence of nine species of edible mushrooms (*Pleurotus squarrosulus, Volvariella volvacea, V. diplasia, Termitomyces eurrhizus, T. microcarpus, Marasmius* sp., *Geastrum* sp., *Calocybe indica* and *Tricholoma lobayense*) in the wild forest in Tripura. Krishnamoorthy (1995) identified a new edible species of *Calocybe* from the sugarcane fields of Coimbatore which was later released as a superior variety APK2 for commercial cultivation. Kalitha *et al.* (1997) reported the occurrence of *Calocybe* in grasslands of Assam. Baroni *et al.* (1999) discovered *Calocybe cyanea* a beautiful agaric in Puerto Rico. Gogoi *et al.* (2000) reported the occurrence of *Calocybe* sp in the moist shady tree bases in Assam. *Calocybe* was also reported from Rocky Mountains of USA by Cripps *et al.* (2002). *Calocybe* are usually found in their natural substrate *viz.*, base of coconut tree, field bunds, base of banyan tree, arecanut fields etc. (Krishnamoorthy and Nakkeeran, 2002). Kalpana (2003) reported the exploration of biodiversity by survey and identified the elite strains of milky mushroom. Survey conducted in Western Ghats by Anandh and Prakasam (2003) resulted in the collection of edible mushrooms *viz.*, *Calocybe indica, Calocybe gambosa, Tricholoma lobayense* and *Tricholoma giganteum.*

2.2 IDENTIFICATION OF NATIVE ISOLATES

2.2.1 Methodology for Identification

This mushroom has been placed in *Calocybe* – Sect I. *Calocybe* (Guttatae) (Fr.) Sing. The hymenophoral trama is regular, with slight divergence below the hymenium. This possess filamentous pileopellis, carminophilous basidia, thin

walled hyaline spores, lack of highly differentiated cystidia and presence of clamp connection. *Calocybe gambosa* differs from *Calocybe indica* in having ellipsoidal basidiospores robust sporocarp and solitary appearance (Purkayastha and Chandra, 1974). *Calocybe indica* belongs to tribus Lyophylleae, family Tricholomataceae of Order Agaricales (Purkayastha, 1985).

Tricholoma, a related species of *Calocybe* was reported from Indogangetic plain during summer season (Chakravarty and Sarkar, 1982). Natarajan and Manjula (1983) observed the occurrence of *Tricholoma lobayense* in Madras. Cultivation of *Tricholoma* on paddy straw was first reported by Ganeshan (1990). Commercial exploitation of an edible tropical mushroom *Tricholoma lobayense* was done by Anandh and Prakasam (2002a).

Tricholoma has a very large pileus 23 cm in diameter, upper surface convex latter flattens with age. Smooth appressed scales at centre, margin incurved, gills decurrent, white alternate hymenophoral trama sub regular, lack of pleurocystidia and chielocystidia. Basidiospores are ellipsoidal, hyaline, smooth and non amyloid (Natarajan and Manjula 1983; Ganeshan, 1990).

2.2.2 Organoleptic Studies

A study conducted by Desai *et al.* (1991) revealed that consumer acceptability of *Pleurotus sajor-caju* was poor due to the tough texture of the stipe and unattractive colour of the pileus but its flavour was found good. In a comparative study Balakrishnan (1994) showed that *Pleurotus sapidus, P. membranaceous* and *P. petaloides* obtained maximum consumer acceptability with respect to colour, appearance and flavour. Overall acceptability of these species were significant compared to the standard species *P. sajor-caju* and *P. flabellatus* which were found inferior in all the qualities.

2.3 ISOLATION AND PURIFICATION

Chandra and Purkayastha (1977) described the tissue isolation technique of *Calocybe* on malt extract agar medium and maintained on the same medium.

Eswaran and Susan (2003) have reported isolation and purification of *Calocybe* on potato dextrose agar medium.

2.4 SCREENING OF MUSHROOMS *IN VITRO*

Suharban (1987) studied the culturing of *Pleurotus* under *in vitro* conditions. Rafique *et al.* (1999) studied the *in vitro* cultivation of *Pleurotus* species on culture media. Yadav *et al.* (2003b) reported the variation in colony morphology of *Agaricus bitorquis* germplasm. The native isolates of paddy straw mushroom (*Volvariella volvacea*) were screened for their nature and radial growth of mycelium (Pramod, 2004).

2.5 SCREENING OF MUSHROOM ACCESSIONS FOR COMMERCIAL CULTIVATION

2.5.1 Spawn

Sinden (1934) was the first to introduce grain spawn for the cultivation of mushrooms. Different kinds of grains namely wheat, rye, millet etc were cooked and mixed with 1:3 per cent weight of $CaSO_4$ and $CaCO_3$. The addition of gypsum and calcium carbonate prevent grains from clogging (Stroller, 1962). Spawn bottles were filled with the substrate and sterilized for 2-3 hrs at 121 °C. The substrate after sterilization contained 40-50 per cent moisture and pH of 7.5. Kostadinov *et al.* (1971) reported the use of liquid spawn mycelium for the production of sporophores in *Pleurotus ostreatus*. Inorganic carrier, perlite has been used for spawn preparation in contrast to organic carriers (Lemke, 1972). Rangad and Jandaik (1977) studied the effect of different spawn substrates and storage condition in the yield of *Pleurotus* spp. They found that freshly prepared spawn produced maximum yield in a number of species of *Pleurotus*. The yield of sporocarp was more or less the same from spawn kept for two months either at room temperature or in refrigerated condition. Thapa *et al.* (1978) devised a cheap and effective method of spawn production in polypropylene covers instead of glass bottles. Spawn production of *Calocybe* have been widely reported on wheat grains (Purkayastha *et al.*, 1981, Purkayastha, 1985). Yield trials of

different strains of *Agaricus bitorquis* indicated that spawn made of jowar grains supported maximum yield followed by bajra grains supplemented with shelled maize cob when used in 1:1 proportion (Guleria *et al.*, 1989). Kotiwaliwale *et al.* (1991) utilised various substrates for spawn production. Cereal maize grain substrate was colonised best in 10-12 days of inoculation. Studies conducted by Mathew *et al.* (1996) on the performance of different species of *Pleurotus* on spawn substrate revealed that sorghum, wheat and paddy grains were equally good for producing spawn.

Krishnamoorthy and Muthusamy (1997a) utilised sorghum grain spawn for *Calocybe* cultivation. Spawn of oyster mushroom prepared on parboiled paddy grains was equally good as spawn prepared in wheat. Spawn prepared from parboiled paddy grains gave 7.50 per cent more yield than conventional cooked paddy grains (Rathaiah and Shill, 1999). According to Balakrishnan and Das (2001) sorghum, wheat or paddy grains are generally used for the preparation of spawn of *Calocybe*. Theradimani *et al.* (2001) used half cooked sorghum grains mixed with $CaCO_3$ at the rate of two per cent for the cultivation of *Calocybe*. Experiment conducted by Thirumalvalavan *et al.* (2005a) to study the various spawn substrates like kudhiraivali, sorghum, sawdust, tea dust and its combination revealed that sorghum and sorghum plus kudhiraivali were the most suitable substrates for *Pleurotus florida.*

Shelf life

Short term storage of white button mushrooms (*Agaricus bisporus)* in perforated and non perforated polyethylene bags at different temperature were studied by Saxena and Rai (1988). The study indicated that mushrooms could be stored in non perforated bags for 4 days at 5 °C, 2 days at 10 °C and 1 day at 15 °C with out veil opening and deterioration. Mehta and Jandaik (1989) reported storage of freshly harvested fruit bodies of *Pleurotus sapidus* on non perforated polythene bags upto 72 hours at room temperature and at low temperature of 0-5 °C upto 15 days. Dhar (1992) observed that *Agaricus bitorquis* had better shelf life when stored in non perforated packs with little change in quality.

Storage in perforated bags resulted in weight loss, veil opening, browning and spoilage. Post harvest washing, treatment with 125 ppm EDTA improved quality as well as shelf life of *Agaricus* (Ahlawat *et al.*, 1998).

2.6 NUTRIENT STATUS

Chang and Chan (1973) observed more protein content in pileus than the volva in *Volvariella volvacea*. Stipe and volva exhibited a more or less similar pattern in protein content and electrophoretic spectra, while the pileus possessed a unique pattern. Certain protein bands existed only in pileus at a specific developmental stage. Purkayastha and Chandra (1976) reported protein content of *Calocybe* mycelium as 19.81 per cent. Among the various aminoacids leucine, threonine, tyrosine and alanine were found predominant in *Calocybe*. Chandra and Purkayastha (1976) reported gain in body weight of mice supplied with mycelial powder of *Calocybe indica*. Nutritive value of *Calocybe* was accounted as 11.90 per cent dry matter, 2.40 per cent protein, 2.25 per cent soluble salts, and 50 kcal of energy by Sivaprakasam *et al.* (1986). Venkateshwarlu *et al.* (1991) noted that volatile flavour compounds of *Calocybe* was attributed to the presence of 1-octen-3-ol, n-octanol, 3-octanol.

Compared to other mushrooms, *Calocybe* have a nutritive value of 4.10 per cent fat, 3.4 per cent crude fibre, and 64 per cent carbohydrate (Doshi and Sharma, 1995).

Krishnamoorthy and Muthusamy (1997a) detected protein content of 32.29 per cent and carbohydrate of 9.85 per cent in milky mushroom. Nutrient content of *Agaricus* consisted of 90.10 per cent moisture, 3.75 per cent protein, 0.53 per cent crude fibre and 4.59 per cent carbohydrate (Singh *et al.*, 1999). Anandh (2001) reported the nutrient composition of *Calocybe indica* with 88.37 per cent moisture, 11.63 per cent dry matter, 26.50 per cent protein, 36.50 per cent fibre, and 8.80 per cent carbohydrate. He also stated the proximate constituent composition of *Tricholoma lobayense* with 85.20 per cent moisture, 14.80 per cent dry matter, 33.20 per cent protein, 23.74 per cent fibre

and 11.38 per cent carbohydrate. Arumuganathan *et al.* (2003) observed total soluble solid content of 5-7 brix in *Agaricus bisporus*.

2.7 CULTURAL STUDIES

2.7.1 Media

Zadrazil (1978) reported malt extract peptone media as a good nutrient media for the growth of *Pleurotus* sp. *Pleurotus eryngii* could grow well on natural solid media such as potato dextrose agar, glucose yeast agar, carrot agar than synthetic medium like Czapeks Dox agar (Sharma and Jandaik, 1984). Suharban (1987) suggested potato dextrose agar as a suitable medium for *Pleurotus sajor-caju, P. florida, P. flabellatus* and *P. ostreatus*. Cultural characterisation of *Lentinus* in various solid and liquid media offered wood extract agar as best solid media followed by potato dextrose agar and glucose asparagine solution as the best liquid medium (Kaur and Lakhanpal, 1999). Out of the eleven culture media evaluated by Rafique *et al.* (1999) potato dextrose agar was found to be the optimum medium for the growth of *Pleurotus*. Similar results were obtained in the case of *Ganoderma* by Balabaskar *et al.* (2005). Ezhilarasi *et al.* (2005) reported potato dextrose agar of pH 5.6 as the best medium for the growth of *Pleurotus sajor-caju*.

2.7.2 Shake Culture Studies

Pleurotus spp. are known to grow well and rapidly under submerged culture. This method has been pointed out by many as suitable for industrial production of mycelium (Stark, 1955; Jennison, 1955). Ramos (1967) reported that addition of coconut milk in a liquid medium enhanced the mycelial growth in the case of *Volvariella volvacea.* Suharban (1987) reported shake culture of different *Pleurotus* spp. in potato dextrose broth for maximum mycelial biomass. Balakrishnan (1994) conducted an experiment to study the effect of coconut milk on the mycelial growth of *Pleurotus* species. The study revealed that oats agar blended with 40.00 per cent coconut milk has supported the maximum growth of all species tested followed by common oats agar medium. Anitha (1998) also

observed enhanced mycelial growth of *Pleurotus* in coconut milk amended medium which was attributed to the presence of kinetin as the growth promoting substance.

2.7.3 Carbon Sources

Madelin (1956) observed starch and glucose as good carbon sources for *Pleurotus flabellatus* and xylose as poor source. Rangaswamy (1956) reported utilisation of starch readily by *Volvariella diplasia.* Litchfield *et al.* (1963) noted disaccharides as best carbon sources for the growth of morel mycelium. Carbon nutrition of *Pleurotus ostreatus* was studied by Yusef and Allam (1967). Studies revealed that maltose supported maximum sporulation of *Pleurotus ostreatus.* Similar results were reported by Bano and Srivastava (1970) in which glucose was considered as a good source of carbon and xylose as poor source for *Pleurotus flabellatus.* Hashimoto and Takahashi (1974) reported xylose and arabinose as poor sources of carbon for *Pleurotus ostreatus.* Starch was found to promote more biomass production of *Pleurotus sajor-caju* followed by maltose, sucrose, and dextrose (Jandaik and Kapoor, 1976). Studies conducted by Chandra and Purkayastha (1977) on the growth requirements of *Agaricus campestris, Calocybe indica, Volvariella volvacea* and *Termitomyces eurrhizus* indicated that carbon sources like mannitol, dextrin, glucose, fructose and sorbitol were best utilised and least utilised were lactose and sucrose. From among the best utilised sources fructose was more or less a good carbon source for all the species mentioned above.

Kikon and Rao (1980) found starch as the best carbon source for *Pleurotus ostreatus.* Kumar and Munjal (1980) recorded maltose, glucose and fructose as better carbon sources than sucrose for *Agaricus bisporus.* Khanna and Garcha (1985) studied the effect of monosaccharides, disaccharides and sugar alcohols for mycelium and protein production of *Pleurotus* sp. Monosaccharide mannose at 2.50 per cent was the most preferred carbon source. Mehta and Kumar (1985) reported maltose as superior carbon source of *Agaricus.* Starch was the best source of carbon

followed by dextrin and xylose for *Pleurotus sajor-caju*. Soluble starch, maltose and glucose were optimal sources for mycelial production of *Tricholoma lobayense*. Highest yield of protein was obtained on maltose and glucose in 5 day old submerged mycelium (Saha and Samajpati, 1987).

Pleurotus platypus formed sporophore primordia in a media containing mannose, trehalose, fructose and sucrose (Bhattarcharjee and Samajpati, 1989). Banerjee and Samajpati (1989) suggested soluble starch and maltose as the best for protein production of *Volvariella diplasia*. Banerjee *et al.* (1990) observed starch as the best exogenous carbon source for the germination and germ tube growth of *Volvariella* followed by maltose, glucose and fructose. Kaur and Lakhanpal (1995) studied the effect of nutrient element sources, vitamins and growth regulator on vegetative growth of *Lentinus edodes*. The study revealed maximum mycelial growth in dextrose followed by fructose and sucrose and minimum mycelial growth was recorded in starch. Results of study conducted by Thirumalvalavan *et al.* (2005c) revealed glucose incorporation on liquid media recorded highest mycelial dry weight of *Pleurotus flabellatus* when compared to dextrose. This was followed by sorbitol, sucrose, cellulose, mannitol and starch in decreasing order of merit.

2.7.4 Nitrogen Sources

Organic nitrogen was superior to inorganic nitrogen for the growth of *Pleurotus ostreatus*. Among the organic nitrogen sources, asparagine was the best (Yusef and Allam, 1967). Nitrates, chloride and tartarate of ammonium supported mycelial growth of *Pleurotus sajor-caju* and *Pleurotus ostreatus* (Bano and Srivastava, 1970; Hashimoto and Takahashi, 1974). Urea was found to be the best source of nitrogen for gasteromycetes. Asparagine at 0.03 per cent concentration supported maximum mycelial growth of *Pleurotus sajor-caju* (Jandaik and Kapoor, 1976). Thianga and Jandaik (1979) recommended use of sodium nitrate as nitrogen source for higher biomass production of *Macrolepiota*. Kikon and Rao (1980) recommended organic forms of nitrogen as suitable source for the growth of *Pleurotus ostreatus*. Khanna and Garcha (1983) tried various

inorganic nitrogen sources namely ammonium chloride, ammonium sulphate, ammonium phosphate, ammonium tartarate, nitrate of potassium, calcium, ammonium and sodium, of which sodium nitrate produced maximum biomass of *Pleurotus sajor-caju* and *Pleurotus ostreatus*. According to Mehta and Kumar (1985) serine proved to be the best organic nitrogen source for the growth of *Agaricus brunnescens*. Mitra and Nandi (1989) observed inorganic nitrogen source ammonium sulphate was the best, producing maximum mycelial biomass on rice straw dust and peptone as the best organic nitrogen source. Complex sources of nitrogen mainly yeast extract, peptone, and casein hydrolysate had more stimulatory effect on protein production of *Volvariella diplasia* (Banerjee and Samajpati, 1989). Peptone was found to be the best for the germination and germ tube growth of *Volvariella* (Banerjee *et al.*, 1990).

2.7.5 pH

Hiroe and Kamyoshi (1937) reported that a pH of 5.0-6.0 was the best for the mycelial growth of *Cortinellus shiitake*. Maximum growth of *Calocybe indica* was obtained at pH 5.5 with 32 mg dry weight (Chandra and Purkayastha, 1977). Maximum mycelial growth of single spore isolates of *Agaricus brunnescens* occurred at a pH ranging from 6 -7 (Mehta and Kumar, 1985). Suharban (1987) suggested pH 5.5 was the best for the maximum dry matter production of *Pleurotus* sp. Best pH for the mycelial growth of Jew's ear mushroom was found to be between 5 - 6 (Guiling and Fuwen, 1988). Banerjee and Samajpati (1989) suggested the optimum pH for the growth of *Volvariella diplasia* to be 6.0. Similar results were given to Banerjee *et al.* (1990). Kaur and Lakhanpal (1999) observed the mycelial growth of *Lentinus edodes* at different pH levels ranging from 3.5 to 8.5 and concluded that acidic pH of 4.5 supported maximum growth. The optimum pH for the growth of *Pleurotus* sp. was found to be 5.5 (Rafique *et al.*, 1999).

2.8 IMPROVEMENT IN PRODUCTION TECHNOLOGY

2.8.1 Screening and Selection

2.8.1.1 Substrates

Purkayastha and Nayak (1981a) reported increased yield when nitrogenous substrates were used for the cultivation of *Calocybe*. *Calocybe indica* could be cultivated on both composted as well as non composted rice straw (Chakravarty *et al.*, 1981). Waste materials like waste paper, sugarcane bagasse, rice straw, wood shavings, coconut waste, and ragi waste were tried as bedding materials for *Pleurotus sajor-caju* cultivation (Sivaprakasam and Kandaswamy, 1981). Among the various agriculture wastes from maize, bajra, mentha, ground nut stalk, cereal straw and vegetable waste, cereal straw was found to be the most suitable substrate for the production of *Pleurotus florida* and *Pleurotus sajor-caju* (Garcha *et al.*, 1983). Experiments conducted by Dadwal and Jamaluddin (1984) reveals barley meal and maize meal as the best substrates for the cultivation of *Tricholoma giganteum.* Combination of alfalfa hay and wheat straw significantly increased total yield and biological efficiency of *Pleurotus sajor -caju* (Royse and Bahler, 1988).

Ganeshan (1990) found fresh paddy straw as a suitable substrate for cultivation of *Tricholoma lobayense.* Oil palm waste when added as a major ingredient in compost of *Agaricus* cultivation resulted in early maturation and harvesting of fruiting bodies (Jimenez *et al.*, 1990). Oyster mushroom cultivated on substrates *viz.,* paddy straw, maize straw, coir dust and groundnut shells, biological efficiency varied widely with maximum in ground nut shell (Desai and Shetty, 1991). Mathew *et al.* (1991) conducted investigation on the utilisation of solid waste from rubber processing as an alternate substrate for oyster mushroom production. Patil and Jadhav (1991) used cotton stalks as one of the best substrate for the cultivation of oyster mushrooms (*Pleurotus sajor-caju).* Sharma and Jandaik (1991) depicted wheat straw, and wheat straw on combination with spent straw as effective substrates in increasing yield of *Pleurotus florida* and *P. ostreatus*. Enhanced yield of *Pleurotus ostreatus* was obtained when the

mushroom was grown on tequila maguey bagasse in Mexico (Velazco *et al.*, 1991a). Sugarcane baggase and cornstover has been utilized as substrate for the cultivation of *Pleurotus* in Mexico (Velazco *et al.*, 1991b).

A noxious weed of tropics, *Chromolaena odorata* when used in combination with paddy straw turned out to be a potential substrate for the cultivation of oyster mushroom (*Pleurotus flabellatus*) (Abraham and Pradeep, 1995). Lime water treated coir waste with paddy straw in 1:1 ratio proved to be a better alternative substrate to conventional substrate for the *Pleurotus ostreatus* cultivation (Eyini *et al.*, 1995). Sangwan and Saini (1995) reported a method of increasing biological efficiency of *Pleurotus sajor-caju* by utilizing a combination of sugarcane bagasse, paddy straw and wheat straw as substrate. Singh *et al.* (1995) proved that sugar cane trash in combination with wheat and paddy straw produced 74.20 per cent biological efficiency of *Pleurotus florida*. Straw of paddy, wheat, linseed, cotton, and jowar were tested for their suitability as substrate for cultivation of *Pleurotus sajor-caju,* of which cotton stalk was the inexpensive and effective substrate (Kathe *et al.*, 1996). Mathew *et al.* (1996) observed a water weed *Eliocharis plantogena* as a potential substrate for cultivation of *Pleurotus citrinopileatus*. Krishnamoorthy and Muthusamy (1997b) utilised several agro wastes *viz.*, paddy straw, sorghum stalks, sugarcane baggase, palmarosa grass, vetiver grass, ground nut haulms, soybean hay and paddy straw compost for cultivation of *Calocybe*. Higher yield and higher biological efficiency was observed in paddy straw followed by maize stalk, sorghum stalk and vetiver grass. Paddy straw compost was not suitable for the cultivation of *Calocybe indica*.

Mixing biomass from *Populus deltoids* and *Eupatorium adenophorum* with paddy straw in 1:2 increased biological efficiency of *Pleurotus sajor-caju* more than hundred per cent (Patrabansh and Madan, 1997). Straw of short duration varieties of rice, *i.e.,* white ponni gave the highest yield of *Pleurotus* cultivation in a study to determine the suitability of various varieties of paddy straw (Eswaran *et al.*, 1998). Geetha and Sivaprakasam (1998) noted a

combination of paddy straw and soybean haulm as a suitable substrate for the cultivation of oyster mushroom (*Pleurotus* sp). A study conducted by Justin *et al.* (1998) revealed that *Pennisetum polystachon* was a superior substrate for the cultivation of *Pleurotus* than common weeds like *Themeda cymbaria, Zingiber zerumbet, Cyprus articulata* and *Typha angustata.* Highest yield of *Pleurotus sajor-caju* was obtained when paddy straw was used as substrate and lowest biological efficiency with betelnut fibre (Chandra *et al.*, 1998; Gupta *et al.*, 1999). Suharban *et al.* (1998) reported pseudostem of red banana as a better substrate for oyster mushroom production when compared to pseudostem of nendran, red banana, palayamkodan, robusta, rasakadali.

Studies by Sivrikaya and Peker (1999) revealed that, forest waste especially wood waste can be used as an alternate substrate for cultivation of Pleurotus *florida.* Upadhyay and Rai (1999) reported the successful cultivation of *Lentinus squarrosulus* on chemically treated wheat and paddy straw. Wild grasses *Pennisetum polystachon Aristada abscendus, Panicum humile, Desmostachya pinnata* when used as substrate in combination with paddy straw resulted in higher yields of *Pleurotus* in West Bengal (Das *et al.*, 2000a). Soybean straw substrate gave higher yields of *Pleurotus columbinus* when compared with the common substrates, wheat straw and paddy straw (Thakur *et al.*, 2000). Combination of linseed straw and ageratum twigs yielded maximum number of sporophores and highest biological efficiency of *Pleurotus sajor-caju* (Kumar *et al.*, 2000). Among the newer substrates like malt industry waste, tea leaf industry waste, dry poplar leaves tried for the cultivation of *Pleurotus sapidus*, and *Pleurotus flabellatus,* malt industry waste rich in nitrogen could replace wheat straw up to 20.00 per cent (Upadhyay and Verma, 2000). Nasrin *et al.* (2001) successfully cultivated *Calocybe* on fermented coir pith with 49.00 per cent biological efficiency. Yadav *et al.* (2001) reported the use of spent mushroom compost for enhancing the grain yield of maize hybrids.

Fermentation of coir pith proved to be an effective pre-treatment to enhance the yield of milky mushroom (Bhavana and Thomas, 2002). Several plant

wastes *viz.*, dried tumeric leaves, sugarcane bagasse, tea waste and java citronella grass were tried as alternate substrates for the cultivation of *Pleurotus citrinopileatus* by Gogoi and Adhikary (2002).They reported dried turmeric leaves giving 93.70 per cent biological efficiency can be used as alternative substrate for oyster mushroom cultivation. Combination of wheat straw and paddy straw was found to be a suitable substrate for the cultivation of *Calocybe indica* (Jadhav *et al.*, 2002). Jain and Vyas (2002) reported utilization of different substrates and their combination to enhance the biological efficiency of *Pleurotus florida*. Wheat straw in combination with soybean straw gave 93.00 per cent biological efficiency. Utilisation of gram straw, sugarcane bagasse, wheat straw and parthenium grass for the cultivation of *Pleurotus flabellatus* has been investigated by Namdeo and Thakur (2002). Suman (2002) investigated on the utilisation of sunflower seed meal and cotton seed meal as substrates for the production of *Agaricus bisporus* which resulted in 32 per cent increase in biological efficiency.

Pandey and Tewari (2003) reported successful cultivation *Tricholoma giganteaum* with paddy straw giving a biological efficiency of 92.00 per cent. Bhavana and Thomas (2003) reported cultivation of nine species of *Pleurotus* on coconut leaf stalk. Acid hydrolysed leaves of certain Indian forest trees, *Butea monosperma*, *Peltophorum ferruginum, Heterophragma adenophyllum*, *Terminalia catappa* and *Polyalthia longifolia* has been utilized for the cultivation of oyster mushroom (*Pleurotus sajor-caju*) (Arya and Arya, 2003).

Among the six different substrates *viz.*, cotton stalk, groundnut haulms, groundnut shell, paddy straw, sorghum straw and sugarcane bagasse, paddy straw was found to be the best substrate for *Calocybe indica* producing the maximum sporophore yield (Eswaran and Susan, 2003). Krishnamoorthy (2003) conducted trials on *Calocybe* variety APK2 and it was observed that the isolate produced maximum biological efficiency of 145 per cent on paddy straw when compared to other substrates. Sharma (2003) reported wheat straw, followed by paddy straw as the best substrate for pink oyster mushroom (*Pleurotus djamor*).

Sherin *et al.* (2004) conducted experiment to study the suitable substrate for *Calocybe indica* cultivation among retted, non-retted and composted coir pith and also paddy straw. The study revealed that maximum yield and sporophores production were observed in non retted coir pith in combination with 75 per cent paddy straw, followed by 50 per cent combination of non retted coir pith and spent mushroom substrate. Custodia (2004) reported coco lumber saw dust as substrate for oyster mushroom cultivation. Combination of hazelnut husk and wheat straw was found to be a suitable substrate for cultivation of *Pleurotus* sp. with 96 per cent biological efficiency (Pekon and Kucukomuzhu, 2004). Highest biological efficiency of *Volvariella* was obtained on oil palm waste when compared to other locally available substrates, dried water hyacinth, banana leaves and sugar cane baggasse (Pramod *et al.*, 2004). Rathore and Thakore (2004) concluded wheat straw as the best substrate for producing maximum number of sporophores and yield of *Pleurotus*.

Umamaheswari and Vijayalakshmi (2004) reported mixture of vermi fertilizer and paddy straw for the successful cultivation of *Calocybe*. Zacharia and Doshi (2004) observed wheat straw as the best substrate for cultivation of *Tricholoma crassa*, an edible ectomycorrhizal mushroom. Magendra and Nageswaran (2005) stated that a combination of water hyacinth and paddy straw in 1:1 proportion increased yield and size of oyster mushrooms. Pramod *et al.* (2005a) observed red banana pseudostem as the most efficient substrate for cultivation of oyster mushroom. Studies conducted by Reddy *et al.* (2005) on various substrates *viz.*, soyabean hull, sun flower stalk, maize stalk, dehulled maize cob, paddy straw, sugarcane bagasse, red gram hulls, and casties stalk, paddy straw gave the highest yield of *Pleurotus florida* and *Pleurotus sajor-caju.* Sangeetha *et al.* (2005) investigated on the efficacy of different substrates for the production of fruiting bodies of *Pleurotus citrinopileatus.* Paddy straw substrate gave maximum yield and biological efficiency followed by banana leaf sheath and coir pith. They also observed that water hyacinth did not support fruiting of *Pleurotus.* Sudhakar *et al.* (2005) reported that combination of horse gram and

capsicum waste gave higher yields of *Pleurotus sajor-caju* and lesser time for spawn run and first harvest.

2.8.1.2 Substrate Sterilisation

Bano *et al.* (1979) reported that hot water treatment at 65 °C for 10-15 minutes was effective in reducing the contamination in *Pleurotus flabellatus* cultivation. Treating paddy straw substrate in hot water at 60-80°C for two or three hours has been recommended for cultivation in *Calocybe* (Purkayastha *et al.*, 1981). Sterilization of substrates by pasteurisation or fermentation process at 40-90°C was effective for mushroom cultivation (Zadrazil and Grabbe, 1981). Soaking paddy straw in clean water for 18-24 hours and subsequently immersing in hot water at 63-80°C for 3 hours has been recommended for cultivation of *Calocybe indica* (Purkayastha, 1985).

Chemical sterilization of the substrates with formalin (200 ppm) and carbendazim (2.5 g) for cultivation of the *Pleurotus* has been attempted by Gokulapalan *et al.* (1989). They also observed that these treatments enhanced yield of mushrooms as well as the sporophore number. Jadhav and Jagtap (1991) reported chemical sterilization of wheat straw in 500ppm formalin and 75 ppm bavistin was effective in producing maximum yield of oyster mushroom. In trials conducted to determine the best substrate sterilization procedure, Singh *et al.* (1991) observed maximum yield of mushrooms on substrate treated with bavistin and formalin. Steam sterilization at 15 lbs for 30 minutes was also effective. Trivedi *et al.* (1991) suggested chemical sterilization of substrate as an effective method of maximising the yield of *Calocybe* and reducing the contaminants. Vijay and Rai (1991) observed absence of residue of carbendazim (75 ppm) and formalin (500 ppm) when used for substrate sterilisation.

Nallathambi and Marimuthu (1994) compared the substrate treatments *viz.*, hot water, steam and chemicals (carbendazim 75 ppm, formalin 500 ppm) on the enzyme activity and yield of *Pleurotus* sp. and reported that chemical treatment was effective in managing contaminants.

Treatment of wheat straw with bavistin 0.10 per cent gave significantly higher yield of *Pleurotus sajor-caju* where as combination of formalin (0.05 %) and dithane M-45 was effective in the case of *Agaricus bisporus* as reported by Chitale and Singh (1995). Krishnamoorthy and Muthusamy (1997 b) suggested boiling of paddy straw bits for 30 minutes in *Calocybe* cultivation. Dhar (1998) concluded that carbendazim, benomyl, mancozeb, and zineb could be used for controlling mould competitors of *Agaricus bisporus*.

Steam pasteurization of pre soaked paddy straw produced the highest number of sporophores and biological efficiency in *Pleurotus* cultivation (Dubey, 2000). Shukla and Biswas (2000) observed that among the different substrate sterilization procedures (hot water method, modified hot water method , chemical .(75 ppm bavistin and 500 ppm formalin) and bavistin 75 ppm alone, the treatment involving 75 ppm bavistin and 500 ppm formalin was effective in producing maximum biological efficiency and weight of sporophore of oyster mushroom.

Patel (2001) reported mulching moistened soil with transparent polyethylene sheet during summer was effective in the management of soil borne pathogens. Moistened spent substrate mixed with rice bran were filled in polyethene covers and steaming for one hour in an autoclave for sterilization in *Calocybe* cultivation has been recommended by Balakrishnan and Das (2001). Theradimani *et al.* (2001) reported pre soaking of paddy straw beds in cold water for four hours followed by boiling for 30-40 minutes as a method for substrate sterilization in *Calocybe*.

Anandh (2001) recommended safe method substrate sterilization for *Tricholoma lobayense* with 100 ppm carbendazim and 500 ppm formalin for 16 hours, since there was no detectable residue of the chemicals. Pandey and Tewari (2002) suggested pasteurisation of straw by hot water treatment or steam for one hour to avoid contamination by micro organisms. Sermkiattipong (2002) suggested radiation pasteurisation with 10 KGy γ rays on rice straw, waterhyacinth, paragrass and manila grass for enhanced faster fruiting body

formation of *Volvariella.* Yadav *et al.* (2003d) tried four different sterilization technique *viz.*, hot water, steam, solar and chemicals for the cultivation of *Tricholoma crassa.* The results indicated that maximum yield of mushroom was obtained in chemical sterilization technique followed by hot water, steam sterilization and the least with solar methods.

Contreras *et al.* (2004) recommended soaking of substrate in alkaline water for 0-48 hrs enhanced yield of *Pleurotus ostreatus* and reduced the fungal contaminants. Bhardwaj and Jandaik (2005) suggested steam pasteurisation of wheat straw as an effective method in sterilization of substrate for *Calocybe indica.* Among different sterilization techniques *viz.*, hot water treatment, autoclaving, formalin and bavistin treatment and different fungicide (bavistin, formalin, benlate, captan and daconil) the best spawn run and yield was observed in substrates sterilised with bavistin 75 ppm and formalin 750 ppm, followed by autoclaving of substrates (Khan *et al.*, 2002). Pandey and Tewari (2003) devised solar pasteurisation as an effective sterilisation technique in the cultivation of *Calocybe indica* and *Tricholoma giganteum.* Effect of various substrate sterilisation techniques on the yield potential of *Pleurotus* was studied by Ram and Thakur (2005). The study revealed that higher yield was recorded from chemical treatment followed by autoclaving of the substrate and solarisation. The radiation techniques also resulted in good yield of *P. florida.*

2.8.2 Substrate Supplementation

Supplementation of compost with cotton seed meal enhanced the yield of *Agaricus* (Beck and Ramussen, 1968; McCanna, 1968). Seth (1976) stated wheat bran as a suitable organic supplement of compost for *Agaricus* cultivation. Addition of peptone and ground nut cake to spawn enhanced yield and number of sporophores of *Pleurotus* (Sivaprakasam and Kandaswamy, 1980). Highest yield of *Calocybe* was obtained when paddy straw was supplemented with five per cent maize meal (Purkayastha *et al.*, 1981). Bano and Rajarathnam (1982) suggested increased yields of *Calocybe* could be obtained by supplementing straw substrate with horse gram powder and yeast mud at 4.40 per cent and 2.20 per cent

respectively with 100 per cent BE. Purkayastha (1985) observed combination of rice straw and wheat straw along with maize meal as suitable substrate for the cultivation of *Calocybe*.

Studies conducted by Gunasegaran and Graham (1987) indicated that among the organic additives like rice bran, corn meal, coconut cake and tobacco dust, rice bran was a suitable additive for increasing the yield of *Pleurotus sajor-caju*. Increasing concentration of rice bran from five to ten per cent resulted in an increase of biological efficiency from 50-59 per cent.

Li *et al.* (1988) reported enhanced yield of paddy straw mushroom by supplementing with five per cent wheat bran, followed by 10 per cent cotton hull. Increased yields of *Pleurotus eryngii* was obtained by supplementation with one per cent wheat bran (Bahram, 1989). Enrichment of rice straw with soybean flour gave the highest yield (79 % BE) of *Pleurotus* (Mahmoud and El-Kattan, 1989). Tan and Chang (1989) reported addition of 10 per cent wheat flour to sawdust media resulted in doubling of biological efficiency of *Lentinus edodes*. Similarly addition of tea leaves to sawdust enhanced yield up to 6-7 folds.

Azizi *et al.* (1990) suggested cultivation of *Pleurotus sajor-caju* on sugarcane bagasse fortified with one per cent ammonium sulphate and 0.50 per cent KH_2PO_4. Bahukhandi (1991) suggested supplementation of paddy straw with organic amendments *viz.*, bran at six per cent and eight per cent resulted in higher yield of *Pleurotus sajor-caju* and *Pleurotus sapidus*. Supplementation of neem cake at five per cent reduced number of days of spawn run and maturity in all *Pleurotus* sp. Concentration above five per cent had detrimental effect on yield (Nallathambi, 1991). Studies conducted by Rao (1991) revealed supplementation of substrate with rice bran and cashew apple waste at four per cent level showed increased yield of *Pleurotus* to the tune of nine to 11 per cent.

Supplementing mushroom beds at spawning with extra organic nitrogen 0.3-0.60 per cent of dry substrate, increased yield in both *Pleurotus florida* and *Pleurotus sajor-caju* (El Kattan *et al.*, 1991). Substrate soaking with five per cent

glucose or sucrose, 0.05 per cent niacin, 50 ppm malic acid resulted in optimum mycelial growth and IAA was the best in promoting fructification (Kadiri, 1991). Paddy straw supplemented with wheat bran recorded high yield of *Pleurotus eous* and lower yield with paddy straw, sugarcane bagasse mixture (Gupta *et al.*, 1991). According to Trivedi *et al.* (1991) the best substrate for the cultivation of *Calocybe* was chemically sterilised wheat straw supplemented with maize meal. Savalgi and Savalgi (1991) demonstrated higher mushroom yields of *Pleurotus florida* , *Pleurotus ostreatus* and *Pleurotus sajor-caju* when cotton waste substrates were supplemented with four per cent rice bran and two per cent soy dal powder.

Supplementation with cotton seed meal at one and two per cent on compost on dry weight basis gave higher yields by 20-30 per cent of *Agaricus* (Gupta and Vijay, 1992). Supplementation of coco-sawdust with corn meal, chicken manure and rice bran enhanced mycelial growth of *Volvariella volvacea* (Matiru and Quimio, 1992).

Geetha and Sivaprakasam (1994) reported neem cake and cotton seed as amendments for enhanced yield of *Pleurotus* sp. They also stated that increase in concentration of organic amendments above four per cent was detrimental to yield. Sharma *et al.* (1994) investigated on the supplements suitable for cultivation of *Calocybe* and found that maize meal, rice husk and coconut husk were suitable for mushroom production. Addition of salts of ferrous sulphate and zinc sulphate also increased the biological efficiency. Kathe *et al.* (1996) observed improved yield of *Pleurotus sajor-caju* with three per cent soybean supplementation.

Neem cake at two per cent rate of supplementation on paddy straw reduced the time taken for spawn run and hastened the maturity of sporophores of *Pleurotus sajor-caju* (Hazarika, 1998). Four agricultural wastes *viz.*, oil palm fruit pericarp fibre, rice husk, melon husk and coconut fruit fibre were supplemented with NPK fertiliser and used as substrate to cultivate *Pleurotus tuber regium.* Oil

palm fruit pericarp fibre at one per cent level gave the maximum yield (Isikhuemhen and Okhuoya, 1998).

Among the different supplements *viz.*, wheat bran, maize grain powder, rice bran, mahua cake and neem cake (5.00 per cent), neem cake supplemented substrate gave maximum yield of *Pleurotus citrinopileatus* and reduced the number of days taken for spawn run (Srivastava and Singh, 1999). Upadhyay (1999) reported higher yields of *Auricularia* with unsupplemented wheat straw as substrate.

Balakrishnan and Das (2001) reported high biological efficiency of *Calocybe* by cultivation on spent mushroom substrate supplemented with 20 per cent rice bran. Supplementation of paddy straw with pigeon pea dal at five per cent on dry weight basis during spawning gave the highest yield and maximum biological efficiency followed by rice bran in the cultivation of *Pleurotus* sp. (Dubey, 2001). Khanna *et al.* (2001) observed enhancement of yield from 4.8-22.3 per cent by the addition of metal ions Cu, Fe, Mg and of which addition of Zn at the time of spawning resulted in higher yield of *Agaricus.* Effect of plant growth regulators on the sporophore yield of *Calocybe indica* has been investigated by Theradimani *et al.* (2001). The study revealed the application of kinetin 100 ppm recorded maximum number of sporophores and also sporophore yield. Wheat straw compost supplemented with soybean cake resulted in maximum yield of *Agaricus* over control (Saharan and Guleria, 2001).

Chemically treated coir pith supplemented with neem cake (10 per cent) or rice bran (10 per cent) increased the yield of *Pleurotus florida* and reduced the time taken for mushroom production (Geetha *et al.*, 2002a). Kumar and Singh (2002) utilised different supplements like wheat bran, rice bran, gram powder, neem cake and chicken manure to produce maximum yield of *Volvariella diplasia.* Chicken manure supplemented paddy straw reduced the number of days taken for spawn run and produced maximum yield (biological efficiency of 22.18 per cent). Post composting supplementation of short method of compost with soybean meal, cotton seed meal and deoiled soybean meal at

different doses at spawning and at casing indicated that supplementation of compost at one per cent fresh weight of compost is beneficial both at spawning as well as at casing for *Agaricus bisporus* (Vijay and Sharma, 2002). Deoiled soybean meal the rate of two per cent of dry weight of substrate resulted in significantly higher yield of *Pleurotus* (Wange *et al.*, 2002). Eswaran and Susan (2003) reported that addition of maize flour (5.00 per cent) to the best substrate namely paddy straw hastened the vegetative growth and sporophore yield of *Calocybe indica.*

Strains of *Volvariella volvacea* were tested for the effects of different organic supplements (cotton seed cake, cotton waste, neem cake, soybean meal, deoiled rice bran, mustard cake, wheat bran and pea nut cake) indicated that rice bran supplementation to paddy straw gave the maximum yield of mushroom (Kaur *et al.*, 2004). Zacharia and Doshi (2004) evaluated effect of different supplements (cotton linter, termitorium soil, coconut husk, dehydrated lucerne, maize meal, rice bran and wheat bran at different concentration on the yield of *Tricholoma crassa.* Maximum fruiting bodies were obtained when supplemented with cotton linter (10 per cent). Sporophores failed to develop in lucerne, maize meal, rice bran, cotton seed, banana pseudostem and wheat bran due to weakened vegetative growth prior to casing.

Thirumalvalavan *et al.* (2005b) used additives (chicken manure, horse gram, soybean and their combination) in enhancing spawn run and sporophore yield of *Pleurotus florida.* Horse gram with chicken manure supported maximum growth of *Pleurotus florida.*

Senthilkumar (2005a) reported ground nut oil cake as a suitable supplement compared to neem cake in maximising the biological efficiency of *Pleurotus eous*, *P. djamor* and *P. sajor-caju.* Additives like chicken manure, farmyard manure, horse gram, soybean and their combinations were tried for the cultivation of oyster mushroom. Paddy straw supplemented with horse gram and chicken manure produced maximum yield of *Pleurotus eous* (480 g), *P. djamor* (462 g) and *P. sajor-caju* (447g).

Effect of micro nutrients on the yield of *Calocybe indica* was studied by Senthilnambi *et al.* (2005b). They reported that among different concentration micronutrients($MgSO_4$, $FeSO_4$, Borax, $CaCO_3$, $ZnSO_4$ at 2, 2.5, 5g) $ZnSO_4$ at 2.5 g mixed with 150 gm red soil applied as casing material gave a maximum yield followed by $CaCO_3$ at 2.5g. Lowest yield was recorded for borax at 5.0 g.

2.8.3 Casing Material

Casing is an absolute requirement for the proper fructication of *Calocybe.*

Purkayastha *et al.* (1981) reported casing material as a mixture of sand, soil (1:1) and 12 per cent $CaCO_3$ as casing material for casing of *Calocybe* beds. Cabutz, the solid fraction of digested slurry from fermentation of cattle manure can be used as casing material for the cultivation of *Agaricus bisporus* (Levanon *et al.*, 1984). Purkayastha (1985) noted dried loamy soil or garden soil (granular) and sand (1:1) with $CaCO_3$ (12 per cent of sand and soil mixtures as an appropriate casing material for *Calocybe*. Trivedi *et al.* (1991) utilised two year old cow dung patties sterilized by two per cent formaldehyde solution for 48 hours as casing material for cultivation of *Calocybe* in the semi arid regions. An ideal casing material should be neutral in reaction, free from disease, pest, competitor organism and undecomposed vegetable matter (Phutola *et al.*, 1991). Supplementation can either be done at the time of spawning or casing but supplementation at casing allows fresh nutrients to reach the *Agaricus* and can offer strong competition of organisms (Kurtzman, 1991). Pandey and Tewari (1994) reported the use of coir dust as a better casing material due to its high water holding capacity and porosity and clean sporophores. Doshi and Sharma (1995) utilized cow dung patties as a good casing material in *Calocybe* cultivation.

Khanna *et al.* (1995) investigated on the suitability of different casing materials (farm yard manure, loam soil, clay soil burnt rice husk, two year old spent compost, and digested biogas slurry) and their combination for casing the mushroom beds of *Agaricus bisporus*. It was observed that burnt rice husk and biogas slurry can be mixed with clay, loam soil which can be used as casing

material. Out of the various casing materials (cow dung patties, biogas slurry, horse dung, moss grass) and their combinations tried for *Calocybe indica* biogas slurry and two year old cow dung patties were found equally suitable with biological efficiency of 98.7 per cent and 100 per cent respectively (Sharma *et al.*, 1997a). Krishnamoorthy and Muthusamy (1997a) reported the use of clay loam garden soil of pH 8.4 as a better casing material in *Calocybe* cultivation. Casing material consisting of sand, soil and cattle dung (1:1:1) was the best for giving higher yield of *Calocybe* (Balakrishnan and Das, 2001). Theradimani *et al.*, (2001) concluded that optimum casing thickness for higher yield was found to be 1-2 cm. Krishnamoorthy *et al.* (2002a) found that clay loam soil of pH 8.4 having 50 per cent moisture as the best casing material for *Calocybe.* They also reported clay soil had moderate bulk density, more pore space, good water holding capacity and certain pseudomonads essential for the fructification of sporophores. The combination of farmyard manure, garden soil and sand (4:2:1) was the best casing material for obtaining higher yield of *Agaricus bisporus* (Raina *et al.*, 2002). Different casing materials were evaluated for maximizing the yield of *Calocybe* by Geetha *et al.* (2002b). Among the various materials coir pith supplemented with $CaCO_3$ gave the maximum biological efficiency, number of sporophores as well as minimum period for sporophore production.

Of the various casing materials evaluated for *Agaricus bisporus* with casing materials prepared from biogas plant slurry, burnt rice husk, farmyard manure, sandy soil and spent compost, the casing material with FYM and burnt rice husk in 2:1 proportion produced maximum yield compared with the control (Angrish *et al.*, 2003). Eswaran and Susan (2003) used casing material comprising of two year old farm yard manure and field soil in 1:1 proportion by weight and sterilised at 80°C for one hour for cultivation of *Calocybe.*

Suman and Paliyal (2004) utilised coconut coir pith, an agricultural waste available in plenty as casing material for *Agaricus bisporus.* They also reported well rotten farm yard manure and coir pith (4:1 v/v) resulted in significant yield increase over control.

Casing materials like field soil, garden soil, farm yard manure, lignite, fly ash and their combination were tried and results indicated that farm yard manure and field soil casing material was the best for giving dense mycelial growth and maximum yield of *Calocybe* (Senthilkumar *et al.*, 2005b). Sterilization of casing material with carbendazim 100 ppm recorded maximum yield of *Calocybe* followed by 50 ppm. Lowest yield was recorded with carbendazim at 250 ppm (Senthilnambi *et al.*, 2005a).

2.9 STRAIN IMPROVEMENT

2.9.1 Irradiation

2.9.1.1 UV Irradiation

According to Trivedi *et al.* (1991) exposure of beds to low light intensity of (150-200 lux) resulted in maximum yield (625 gm) and maximum number of sporophores of *Calocybe indica*. Strain improvement through irradiation has been reported by Geetha and Sivaprakasam (1996). Increased yields of *Calocybe* was obtained at relatively higher light intensities of 1600 lux (Krishnamoorthy and Muthusamy, 1997b). In the study conducted by Theradimani *et al.* (2001) it was observed that the growth chamber with blue coloured high density polyethene sheet roofing material and with blue incandescent light significantly increased sporophore yield of milky mushroom. Nambi *et al.* (2002) observed increased yield of *Calocybe indica* by exposure of spawn bottles to UV rays for 45 minutes.

2.9.1.2 Gamma Irradiation

Staden (1967) observed that irradiation of mushrooms with Υ rays upto 250 KR had better flavour than untreated mushrooms. Langerak (1972) also recommended irradiation of mushrooms from 150-250 KR for prolonging the shelf life of *Agaricus*. Menniti (1976) observed doses of 600 KR were the best for prolonging storage life of *Agaricus campestris*. Roy and Bahl (1984) conducted experiments to determine the effective dose of the γ irradiation (250,400, 550 k rads) in preventing cap opening of *Agaricus bisporus*. Irradiation at all doses prevented increase in button cap diameter, stalk length and deterioration.

Irradiation of *Agaricus* at 100 KR of γ rays was effective in controlling the deterioration of fresh mushrooms (Beelman, 1988).Bahukhandi and Munjal (1988) observed that irradiation of spores of *Pleurotus sajor-caju* had no effect on the number of colonies, but a progressive increase in exposure to γ rays (15 minutes) resulted in the decreased number of colonies. Roy and Bahl (1989) studied effect of different doses of γ irradiation on the cap opening of *Agaricus*. Weight loss and cap openings were delayed at 400 KR. Attempts made by Anitha (1998) to develop mutants of *Pleurotus* spp. by gamma irradiation showed enhanced yield in *Pleurotus florida* at 2.5 KR, Ananthan at 1.5 KR. Sporocarp yield of *P. platypus* increased as the level of irradiation was increased. Sarala (2001) reported γ irradiation of cultures at 1KR took less time for first harvest, and with maximum biological efficiency in the case of *Pleurotus*. Exposure of spores of *Pleurotus florid*a to 1000, 2000, 3000, 4000, 5000 rads showed maximum yield at 3000 rads and above that there was reduction in yield (Ravichandran and Muthusamy, 2005). Pramod *et al.* (2005b) also reported enhanced yield of *Volvariella volvacea* by irradiation using γ rays at 1000 rads.

2.9.2 Mating/ Crossing

2.9.2.1 Multi Spore Crossing

Bhandal and Mehta (1989) reported that the growth was invariably slower in tissue culture and tend to yield lesser than the parent cultures. Variation due to the anastamosis of multispore isolates were less hence not widely used in breeding programmes.

2.9.2.2 Single Spores

Eger (1978) described the procedure of single spore isolation and preparation of monospore cultures. Single spores were selected from agar plates under the microscope and transferred to separate plates. In India the first attempt at raising of single spores was made by Kumar and Munjal (1980). They selected 30 single spore isolates on the basis of rate of growth and the isolates gave 12.3-21.98 per cent increased yield over the parent strains. Lo and Chen (1989) devised

a technique for the isolation of single spores using serial dilution and locating it by means of objective of a dissection microscope. Suman (1993) reported the occurrence of strandy and silky type of mycelial growth with a growth rate faster than that of the parent in the case of *Agaricus bisporus*.

2.9.2.3 Hybridisation

Ghosh and Chakravarty (1991) conducted studies on evolving new strains of *Pleurotus sajor-caju* by selective dikaryotisation. Nine monokaryons of selected parents were crossed resulting in dikaryon formation with improved qualities like crop earliness, reduced percentage of bud mortality, size, shape, and colour of pileus. Thakur and Bhandal (1992) attempted hybridization of non fertile isolates of *Agaricus bisporus*. Crossing of strains with good yield, but poor quality and other strains with normal yield resulted in crosses better than parental strains.

Phutela and Garcha (1995) isolated monospores of *Agaricus bisporus* from parent S11 having better yield performance on wheat straw compost. Pahil *et al.* (1999a) conducted trials on monokaryon of *Agaricus bisporus* and concluded that the fruiting bodies of homokaryons were normal and mycelial growth was well established, but the number of fruiting bodies was less. Pahil *et al.* (1999b) conducted studies on dikaryotic and homokaryotic stocks of tropical *Agaricus bitorquis*. Homokaryons generally have slow and dense mycelial growth when compared to dikaryon having faster and stranded mycelial growth. Homokaryons of tropical *Agaricus bitorquis* produced abundant fruiting bodies on composted substrate. Monospore culture of *Agaricus bitorquis* had fine fluffy and silky growth with appressed strandy type mycelia (Reena *et al.*, 1997). Verma *et al.* (2000) reported strain improvement of mushrooms through multi spore culture single spore isolation and hybridisation. Salmones and Duran (2001) reported crossing of monokaryons (*Agaricus*) with a yield potential of 8.4-25 per cent to produce dikaryon having early fruiting body and yield potential of 19-30 per cent. Periasamy and Natarajan (2002) attempted mating of single basidiospore isolates resulted in the formation of dikaryons with better

biological efficiency, enriched proteins and minerals. Yadav *et al.* (2002) isolated single spores of high yielding species of *Agaricus bisporus* which were having slow mycelial growth and stranded mycelium. Single spore isolate produced fruiting bodies with short stipe and pink gills, whereas recombinant lines produced mushrooms with broad stipe and poorly developed gills. Hyphal anastamosis and mating of dikaryons of *Pleurotus djamor* var. *roseus* gave hybrids with higher biological efficiency and less spawn run periods (Periaswamy and Natarajan, 2003). Yadav *et al.* (2003c) exploited germ plasm of *Agaricus bitorquis* for developing heterokaryotic inter strainal hybrids having good traits and disease resistance.

2.9.2.4 Enzyme activity

Royse and May (1982) used isozyme variations in mannose phosphate isomerase, peptidase, alcohol dehydrogenase, and glutamate pyruvate transminase to identify gene type classes of *Agaricus brunnescens.* Enzymatic degradation of the substrate is usually associated with phenol oxidase activity of the mushroom (Sermanni *et al.*, 1985). Doshi *et al.* (1987) assayed the pectinolytic enzymes like polygalacturonase, polymethylgalacturonase, polygalacturonase transeliminase, pectin transeliminase , and cellulase from spawn bottle of *Calocybe* and observed activity in the order PG> PMG>cellulose>PGTE>PTE after 25 days of mycelial growth.

Kannan and Oblisami (1990) reported that polyphenol oxidase activity in *Pleurotus sajor-caju* tremendously increased in the initial stages and associated with growth.

Addition of casein and copper sulphate resulted in increased enzyme yield from enzyme mixture of *Agaricus bisporus* that contain polyphenol oxidase and laccase (Grabbe, 1993). *Calocybe indica* was having least activity of cellulase and laccase activity compared to *Pleurotus* (Ramamoorthy *et al.*, 1999). Packia *et al.* (2000) demonstrated genetic variability of mutant strains of *Pleurotus citrinopileatus* based on enzyme electrophoresis. Anandh and Prakasam (2002 b) noted the increase in cellulose activity of *Tricholoma lobayense* up to

second harvest which are associated with initiation of mushroom flushes. Laccase and polyphenol oxidase activities attained peak during mycelial growth and decreased during sporophore initiation.

Depending upon the substrate, enzyme produced varied in *Calocybe indica* as cellulose activity was more on paddy straw, sorghum stalks, maize stalks etc, where as black gram hay and soyabean hay recorded higher laccase activity. Fungus colonised sawdust and coir pith compost enhanced the activity of poly phenol oxidase (Krishnamoorthy *et al.*, 2002b). Owseph *et al.* (2003) observed that maximum production of cellulase and laccase enzyme was on paddy straw substrate twenty days after inoculation of *Pleurotus sajor-caju.* Krishnamoorthy *et al.* (2005b) observed variation in enzyme production *viz.*, polyphenol oxidase, cellulose, laccase depending upon the variation in substrates.

2.10 MOLECULAR CHARACTERISATION

Purkayastha and Nayak (1981b) reported that mature fruit bodies of *Calocybe* contained greater amount of proteins than button. They also observed that gel electrophoretic analysis of soluble proteins of fruiting bodies of different developmental stages reveals 20, 18 and 16 protein bands in button, stripe and pileus. Proteins profile studies conducted by Vineeta *et al.* (1998) revealed presence of 18 protein bands in pileus and stipe of each strain of *Agaricus*. Bands were approximately 14-66 KDa, indicating proteins of smaller molecular weight.

Packia *et al.* (2000) evaluated genetic variation of seven mutant lines of *Agaricus* using poly acrylamide gel electrophoresis. Results indicated that the total gene diversity values were considerably higher than the parental strains. Singh *et al.* (2000a) used single primers to generate random amplified polymorphic DNA from commercial strains of *Agaricus bisporus, Pleurotus flabellatus, Pleurotus sajor-caju, Pleurotus ostreatus, Auricularia polytricha* and *Volvariella volvacea.* Of the different primers OPA-01amplified DNA of all the seven strains tested. All the random primers could distinguish mushroom strains with in the species and species with in the genus and different genera. Yadav *et al.* (2002) reported the use of RAPD analysis to confirm the homokaryotic nature

and hybridisation between compatible homokaryon of *Agaricus bisporus*. Singh *et al.* (2003a) have attempted characterisation of edible mushrooms *viz.*, *Agaricus*, *Pleurotus flabellatus*, *Auricularia polytricha* and *Morchella esculenta* using RAPD with random primers. Singh *et al.* (2003b) conducted molecular characterisation of speciality mushrooms like *Volvariella, Lentinula,* and *Calocybe* using RAPD with eight primers. This revealed intergeneric, inter and intra specific variation of each mushrooms. Verma *et al.* (2003) reported characterisation of homokaryons of *Agaricus bisporus* using RAPD and amplifications were done using O and N series primers. They observed lesser band polymorphism in white strain and brown strains of *Agaricus bisporus* reflecting more homogeneity. Yadav *et al.* (2003a) utilised RAPD as sensitive tool for the assessment of genetic variation at DNA level among *Agaricus bisporus* strains and selected diverse parents for breeding programmes. They also showed high degree of genetic diversity between the brown and white strains. Yadav *et al.* (2003b) utilised RAPD markers assisted in selection of genetically diverse parents of *Agaricus bitorquis*. They observed 100 per cent polymorphism in different strains with OPN-01, OPN-02, OPN-05, OPN-06, OPN-07, OPN-08, OPN-09, and OPN-10 primers.

2.11 CONTAMINANTS

Chakravarty *et al.* (1982) reported the inhibitory action of carbendazim at 25 ppm against the contaminants of oyster mushrooms .It was also reported by Vijay *et al.* (1986). Heavy contamination of *Trichoderma viride* in steam pasteurised straw reduced the mushroom yield which was controlled by using formalin and carbendazim (500ppm and 75 ppm) solution for sterilisation of straw (Vijay and Sohi, 1987).

Vijay and Sohi (1989) reported fungal competitors, *Trichoderma viride, Cephalosporium asperu, Cochliobolus spicifera, Drechslera bicolour* and *Phialophora*, reduced the growth of *Pleurotus* spp. by 10 to 100 per cent. Doshi *et al.* (1991) observed competitor moulds like *Trichoderma viride, Sclerotium rolfsii, Aspergillus flavus*, on beds of *Calocybe* and managed them by

the use of carbendazim 50 ppm and Blitox 50 ppm. Of the undesirable fungi *viz.*, *Trichoderma harzianum, T. longibrachiatum, Chaetomium globosum* and *Epicoccum nigrum* on *Agaricus bisporus* bed, maximum yield loss of 50 per cent was due to *Trichoderma* sp. (Tewari and Singh 1991). Sharma *et al.* (1991) reported 45 per cent yield loss by *Trichoderma viride* and two per cent loss by *Coprinus* incidence in *Agaricus bitorquis* cultivation. Sharma and Vijay (1995) reported severe yield loss on *Agaricus bisporus* by *Coprinus*. Sharma and Vijay (1996a) observed high incidence of *Trichoderma viride* on steam sterilised paddy straw for *Pleurotus* cultivation resulting paddy straw 45 per cent yield loss. Sharma and Vijay (1996 b) surveyed on the incidence of brown plaster mould (90.00 %) false truffle (1-90.00 %) green mould, yellow mould and wet bubble. They also observed that competitor and parasitic moulds on casing material and compost includes *Papulospora byssina, Trichoderma, Verticillium, Trichothecium, Coprinus, Chaetomium, Fusarium, Cladobotryium.* Efficacy of formaldehyde fumes against competitors like *Sepedonium chrysospermum, Papulospora byssina* and mycoparasite was studied by Sharma *et al.* (1997b). Exposure of the culture of these moulds to formaldehyde four per cent for 6-24 hours inhibited the growth of the pathogen. Dhar (1998) observed that carbendazim, benomyl, manocozeb and zineb could be best used for controlling the mould competitors of *Agaricus bitorquis.*

Among the different species of *Trichoderma, Trichoderma harzianum* occurred widely than *T. viride* (Jandaik and Guleria, 1999). Anandh *et al.* (1999) studied the yield loss in *Pleurotus eous* due to the incidence by common contaminants *Trichoderma harzianum, Aspergillus flavus* and *Aspergillus niger.* Studies revealed loss of yield ranged from 70-77 per cent. Pani (2000) noted *Coprinus* to be a serious contaminant causing 80 per cent reduction in yield followed by *Sclerotium rolfsii* (74 per cent). Thakur *et al.* (2001) observed that the incidence of fungal competitors was highest during May-July and minimum during January–March. Pandey and Tewari (2002) described green mould, *Chaetomium* and *Coprinus* as the major problematic weed moulds in mushroom cultivation. Singh and Sharma (2002) reported occurrence of *Mycogone perniciosa,*

causal agent of wet bubble disease in various mushroom growing units in Solan. Loss in yield to *Mycogone perniciosa* was 100 per cent in *Agaricus* cultivation which was controlled using sporogon at 0.075.

Seshagiri and Eswaran (2002) observed that inoculation of *T. harzianum,* and *A. flavus* on *Calocybe* beds after casing increased the yield of *Calocybe*. Studies were conducted by Pandey *et al.* (2003) to investigate the occurrence of competitor moulds and pathogens during the cultivation of *Calocybe indica*. *T. harzianum* was the most problematic weed mould during spawn run and cob web disease causing complete crop loss could be managed by carbendazim (0.01 %). Siddique *et al.* (2004) suggested plant derivatives especially onion had maximum inhibitory effect on *Trichoderma* followed by *Aegle marmelos*. Bhardwaj (2005) observed incidence of *T. harzianum*, *Coprinus*, *Papulospora byssina* in *Calocybe* beds which could be managed by carbendazim at 50ppm. Krishnamoorthy *et al.* (2005a) conducted survey on the incidence of pest and diseases of milky mushroom and observed the incidence of *Coprinus* from spawn run till harvest. Raman *et al.* (2005) reported *Aspergillus niger, Aspergillus flavus* and *Trichoderma* sp as major contaminants of *Calocybe* spawn.

2.12 PESTS

Kumar and Sharma (2000) reported phorids as major pests affecting mushrooms. Kumar and Sharma (2001) studied on the seasonal abundance of mushroom pests, indicating presence of phorids and sciarids through out the year in cropping rooms. Pandey and Tewari (2002) suggested management of phorids and sciarids by hot water treatment at 80°C or steam pasteurisation for one hour. They also suggested proper aeration, use of nylon nets, and use of yellow light traps as other management strategies. Deepthi *et al.* (2003) reported for the first time another devastating pest of oyster mushroom *Scaphiosoma nigrofasciatum*. Snail was reported as a new major devastating pest of milky mushroom responsible for severe yield loss by Heera *et al.* (2006).

Materials and Methods

3. MATERIALS AND METHODS

3.1 SURVEY AREAS

During the study period, survey was conducted in the places covering different districts of Kerala *viz.,* Thiruvananthapuram, Kollam, and Pathanamthitta. The forest ranges, meadows, and fields were surveyed regularly during the rainy and post rainy days for the collection of mushrooms. The collected specimens were brought to the laboratory packed in paper covers and subjected to various studies.

3.1.1 Collection of Mushroom Flora

The collected mushrooms were given accession codes and identified. The colour, macroscopic and microscopic characters of collected mushrooms were noted. The native mushrooms were isolated and then subjected to screening process. Those selected after screening were studied further and identified. Studies were conducted based on data sheet as described in Appendix I. The isolates were given accession numbers from National Research Centre for Mushroom, Solan starting from OE-349 to OE- 358.

3.2. IDENTIFICATION OF NATIVE ISOLATES

The mushrooms were screened for their macroscopic details such as shape, size, colour of basidiocarp and microscopic details.

3.2.1 Methodology for Identification

3.2.1.1 Microchemical Reagents

Micro chemical reagents often enables to examine and identify mushrooms and used for distinguishing the like and alikes within the mushroom group.

3.2.1.1.1 Melzer's Reagent

One and half grams of iodine, 100mg of chloral hydrate and 5 g of potassium iodide were mixed with 100ml of water. The water should not be boiled or mixed with alkali. Production of blue black colour by spores on treatment with this reagent indicates amyloid nature of spores. Reddish brown indicates dextrinoid nature and yellow non amyloid.

3.2.1.1.2 Cotton Blue Stain

The stain was prepared by combining 50ml of one per cent solution of cotton blue in lactic acid (100g), phenol (100g), glycerine (150ml) and 50 ml water. The cotton blue turns spore wall of cyanophilic agarics into blue or dark blue.

3.2.2 Screening of Isolates for Organoleptic Characters

The ten isolates were subjected to studies of organoleptic characters like colour, appearance, texture and flavour. The isolates were subjected to evaluation by 10 judges based on a score card and subjected to Kruskal Wallis statistical test for analysis. The average ranking was given for each isolate for each character. Score card values for each character are given in Appendix II.

3.3 ISOLATION AND PURIFICATION OF CULTURE

3.3.1 Tissue Culture Technique

The milky mushrooms obtained from different parts of Thiruvananthapuram, Kollam and Pathanamthitta were isolated using standard technique for tissue isolation. The mushrooms were cleaned up with water to remove adhering soil particles. A fresh mushroom was surface sterilised using ethanol. In the laminar flow chamber, the mushroom was longitudinally split into two equal halves from the pileus to stipe and a small piece of the tissue was removed with a sterile scalpel from the centre of the juncture of the pileus and stipe. The tissue was placed into petri dish containing potato dextrose agar medium (PDA) under aseptic condition. The dishes were incubated at room

temperature (28 ± 2°C) for seven days. The initial growth was transferred into PDA slants and purified by hyphal tip method.

3.4 SCREENING OF MUSHROOM ACCESSIONS UNDER *IN VITRO* CONDITION

The mushroom cultures obtained during the survey were assayed for their growth under *in vitro* condition on petri dishes. The culture disc of 5mm diameter cut out from seven day old culture of each isolate was used for inoculation on potato dextrose agar medium poured in sterile petri dish. These dishes were incubated at room temperature (28 ± 2° C). Three replications were maintained for each isolate and colony diameter was measured at seven days intervals for 14 days.

3.5 SCREENING OF MUSHROOM ACCESSIONS FOR COMMERCIAL CULTIVATION

The mushrooms collected, isolated and maintained on PDA slants in laboratory and were inoculated in paddy grain based spawn material for further study.

3.5.1 Preparation of Spawn

Spawn was prepared as per the method described by Sinden (1934). The paddy grains were used for the preparation of spawn. The grains were cooked for one hour in boiling water. The excess water was drained off and the grains were spread on a clear area. Glucose drip bottles were filled with cooked grains after mixing with calcium carbonate at the rate of 50g kg^{-1} seed. The filled bottles were sterilised at 1.02 kg cm^{-2} pressure for two hours in an autoclave. The mycelial bits from seven day old actively growing pure culture of *Calocybe* sp were inoculated aseptically and incubated at room temperature (28 ± 2°C). Three replications were maintained in each case and mycelial growth of fungi were measured and recorded. The spawn thus obtained as mother spawn was used for further spawn production and also to raise beds.

3.5.2 Screening Trials

Cultivation of different isolates was done by using paddy straw. Substrate was prepared by soaking paddy straw in a solution containing carbendazim (75 ppm) and formalin (500 ppm) for 18 hours. After draining excess water and air drying, the straw was used for mushroom bed preparation. The beds were prepared as per the polybag method described by Baskaran *et al.* (1978). The polythene bags of 60 x 30 cm were used for mushroom bed preparation. The paddy straw bits were placed as twist in bags and spawn laid along the periphery. This was repeated thrice by the application in three layers. The polythene bags were made compact tied at the top, and kept in incubation chamber at 80 per cent relative humidity. Few small holes plugged with cotton were made to facilitate aeration and preventing the entry of insects. After 21 days of spawn run casing was done using sand, soil and calcium carbonate (1:1:0.18 w/w). Regular sprinkling of water was done. The best isolate was selected based on morphological characters like earliness in sporophore production, weight, height of the mushroom, low mortality percentage, and fibre content and over all acceptability.

3.5.3 Screening for Post harvest Quality

An experiment was conducted to determine the shelf life of *Calocybe*. The mushrooms were kept at room temperature and also in refrigerated condition. In room temperature and refrigerated condition, it was kept in open condition, in poly propylene cover (without perforation), and poly propylene covers (with perforations). After shelf life period of 5 days the mushroom which retains the firmness, colour and texture was selected for further studies.

3.6 ESTIMATION OF NUTRIENTS/ PROXIMATE CONSTITUENTS.

3.6.1 Estimation of Moisture Content

Ten gram sample was dried in an oven until constant weight obtained. The initial and final weights were noted. The difference between these two gives the result which is converted into per cent.

3.6.2 Estimation of Total Soluble Solids

One gram each of the pileus and the stipe of the mushroom was ground thoroughly until a few ml of the extract was obtained. One ml of the extract was placed on the pocket refractometer and the reading obtained was read through the instrument. The total soluble solids was measured either in brix or in per cent

3.6.3. Estimation of Crude Fibre

One gm of filtered dried sample was boiled with 100ml of concentrated sulphuric acid (1.25 %) for 30 minutes, by adding bumping chips. The digested sample was filtered through a muslin cloth and washed with boiling waste until the washings were no longer acidic. The sample was boiled with 100ml sodium hydroxide (1.25 %) for 30 minutes. The digested samples were filtered through a muslin cloth and washed with boiling water until the washings were not alkaline. The sample was washed with 25 ml of 1.25 per cent boiling sulphuric acid, 50 ml of water and 25 ml of alcohol. The residue were removed and transferred to pre weighed ashing dish. The residue was dried till a constant weight was obtained.

$$\text{Per cent fibre} = \frac{\text{Loss in Weight}}{\text{Weight of the sample}} \times 100$$

3.6.4 Estimation of Proteins

Protein content of different isolates of mushrooms was estimated using the method described by Bradford (1976).

One gram sample was ground in 10 ml of 0.1 M acetate buffer (pH 4.7). The materials were centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant obtained was used for further analysis. The reaction mixture consisting of 0.5 ml enzyme extract, 0.5 ml of distilled water and 5ml of Coomassie brilliant blue G-250 was used. The reaction mixture was assayed for the absorbance at 595 nm against a reagent blank. Standard graphs were prepared using the bovine serum albumin. Using the graph, the protein content was

determined as microgram albumin equivalent of soluble protein on fresh weight basis.

3.6.5 Estimation of Total Sugars/ Carbohydrates

Total carbohydrate content was estimated by anthrone method (Hedge and Hofreiter, 1962).

One hundred mg of mushroom mycelia was weighed and transferred into boiling tubes. It was hydrolysed by keeping it in a boiling water bath for three hours with 5ml of 2.5 N hydrochloric acid, cooled to room temperature and neutralised with sodium carbonate until effervescence was ceased. The tissue was ground and volume made up to hundred ml and centrifuged at 5000 rpm for 15 minutes. The supernatant collected, was used as an aliquot for analysis. From the supernatant 0.5 ml aliquot was taken and made upto one ml by adding distilled water. The reaction mixture containing 0.5 ml of aliquot, 0.5 ml distilled water and 4ml of anthrone reagent was added to the tubes and heated for 8 minutes in boiling water bath. The reaction mixture was cooled and colour read at 630 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). The amount of carbohydrate present was calculated from standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of sample on fresh weight basis.

3.7 CULTURAL CHARACTERS OF SPECIES OF *CALOCYBE*

3.7.1 Evaluation of Growth of *Calocybe* sp on Various Solid Media

The experiment was attempted to find out the best medium for radial growth of *Calocybe* spp.

Five different solid media *viz.*, potato dextrose agar, malt extract agar, oat meal agar and natural media like jackfruit seed kernel agar, and coconut milk agar were used. The compositions of the media used are given in Appendix III. The media were prepared and sterilised by autoclave at 15 lbs pressure for 15-20 minutes. After cooling it was poured into sterile petri dishes of nine centimetre diameter and allowed to solidify. The culture disc of 5 mm diameter cut out from

seven day old culture of fungus was used for inoculation. The culture disc of the mushroom was inoculated into petri dish which was later incubated at room temperature (28 ± 2° C). Three replications were maintained for each treatment and colony diameter, nature of mycelial growth were measured at weekly intervals for 14 days.

3.7.2 Growth of *Calocybe* in Shake and Static Culture

The different media *viz.*, potato dextrose, oat meal and malt extract broths were used along with jackfruit seed kernel broth and coconut milk broth. The composition was same as used in the previous experiment except for the omission of agar-agar.

The liquid media were prepared and 100 ml of each medium was dispensed in 250 ml conical flask and autoclaved at 1.02 $kgcm^{-2}$ pressure for 20 minutes. The media were then inoculated with 5mm culture disc of fungus, taken from actively growing culture under aseptic condition. The flasks were kept at room temperature for one month. Two sets of flasks were kept in rotary shaker (150 rpm), and another set under static condition for one month. After one month the mycelia were filtered through a Whatman No: 1 filter paper and dried in an oven at 60 °C. The dry weights were taken until a constant weight was obtained.

3.7.3 Effect of Different Carbon Sources on the Growth of *Calocybe* sp.

Calocybe was grown in media with different carbon sources *viz.*, sucrose, lactose, fructose, galactose, mannitol and inositol. These were substituted for dextrose, in potato dextrose medium without agar-agar. Fifty ml of each medium was then inoculated with 5 mm culture disc of actively growing culture and incubated at room temperature (28 ± 2 °C). The mycelial mat was filtered after two weeks and dry weight was taken after drying at 70 °C till a constant weight was obtained. Three replications were maintained in each case.

3.7.4 Influence of Different Nitrogen Sources on the Growth of *Calocybe* sp.

Different forms of nitrogen as ammonium nitrate, ammonium carbonate, ammonium chloride, sodium nitrate, potassium nitrate, beef extract and peptone

were substituted in Czapecks medium so as to give the same per cent of nitrogen in each case. Fifty ml of medium was taken in each 250 ml Erlenmeyer flask sterilised in an autoclave inoculated and incubated at room temperature for 14 days. The mycelial mat was filtered through a Whatman No:1 filter paper and dry weights were taken after drying at 70 °C until constant weight was obtained . Three replications were maintained in each case.

3.7.5 Effect of Different Hydrogen ion Concentration in the Media on the Growth of *Calocybe* sp.

Potato dextrose broth was prepared and pH was adjusted to 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 by adding 0.1N hydrochloric acid or 0.1N sodium hydroxide. Fifty millilitres of each medium was taken in 100 ml conical flask and autoclaved at 1.02 kg cm^{-2} pressure for twenty minutes. The medium was then inoculated with a 5 mm disc of seven day old culture of *Calocybe* and incubated at room temperature (28 ± 2 °C) for two weeks. The mycelial mat was filtered, dried at 70 °C until constant weights were obtained. Three replications were maintained in each case.

3.8 IMPROVEMENT OF PRODUCTION TECHNOLOGY

3.8.1 Screening and Selection of Substrate based on Different Sterilisation Procedure

The best isolate among the ten collected mushrooms was tested for the comparative efficacy for cultivation under Kerala condition. The study was conducted to find out the biological efficiency by using different substrate subjected to different sterilisation procedures. Bed was raised following the poly bag method as described by Baskaran *et al.* (1978). In modified technique, paddy straw made into small twists were used for laying beds instead of paddy straw bits. The different substrates used for the cultivation of mushroom included paddy straw, spent mushroom substrate and coir pith. Spent Mushroom Substrate (SMS) was obtained as a waste material after cultivation of *Pleurotus*. The various substrate sterilisation procedures adopted included boiling in hot water at

100 °C for 1 hour, chemical sterilisation using carbendazim and formalin, at 75 ppm and 500 ppm respectively and solarisation.

For boiling the substrates like paddy straw, coir pith and SMS were soaked in water overnight, taken out, excess water removed and boiled for about one hour. They were then air dried and used for layout of beds.

In chemical sterilisation the substrates were soaked overnight in water containing 75 ppm carbendazim and 500 ppm formalin. The excess water was drained off and spread over a clean area for drying. Such substrates were used for laying out beds

For solarisation purpose high density polyethene sheets of 200 gauge thickness were used. The substrates were soaked in water for 18 hours after draining out the excess water the same was spread over a clean area. The substrates were spread in 10cm thickness layer on the polythene sheet. Further the same was covered with another sheet of same thickness and stapled all over the edges to maintain high temperature developed by the vaporisation and condensation of water (Plate 1a and 1b). The substrates were subjected to solarisation under direct sunlight for three days. The temperature of the substrates during solarisation was also taken. The sterilised substrates were used for bed preparation. Polybag method of cultivation was adopted. The substrates, paddy straw and spent mushroom substrates as twists and coir pith as a layer was placed in polybags. Layer spawning was done along with the periphery which was repeated 4 times. Polythene bags were made compact, tied at the top, and also provided few holes for air circulation. The spawned bags were then transferred to an incubation chamber with 80 per cent relative humidity for spawn run. After completion of spawn run the beds were cased with sand, soil and calcium carbonate (1:1:0.18 w/w). The experiment was laid in Factorial CRD with five replications. The treatments were:

Plate 1a Solarisation

Plate 1b Close up view of solarised substrate

EXPERIMENT 1

Treatments

T_1 - Paddy straw + Boiling

T_2 - Paddy straw + Solarisation

T_3 - Paddy straw + Chemical (Carbendazim 75 ppm and formalin 500 ppm)

T_4 – Spent mushroom substrate + Boiling

T_5 - Spent mushroom substrate + Solarisation

T_6 - Spent mushroom substrate + Chemical (Carbendazim 75 ppm and formalin 500 ppm)

T_7 – Coir pith + Boiling

T_8 - Coir pith + Solarisation

T_9 - Coir pith + Chemical (Carbendazim75 ppm and formalin 500 ppm)

The best treatment among these were selected based on the criteria like time taken for spawn run, number of sporophores produced , total yield, incidence of pest, diseases and competitor moulds. The best substrate and its sterilization procedure were used for standardisation of production technology of milky mushroom.

3.8.2 Effect of Supplements on the Yield

The supplements used for the experiment includes rice bran, neemcake, vermicompost and micronutrient, ferrous sulphate. The sterilized substrate was used for bed preparation. The supplements rice bran, neem cake and vermicompost were mixed @ 20 g/kg and 40 g/kg substrate for laying out the beds. Ferrous sulphate was also added @ 5 ppm and10ppm in the beds. The beds were laid in polyethene bags of 60 x 45 cm using the substrate. Spawning was done along the periphery. Four layers of the substrate were laid. The polyethene bags were made compact tied at the top, and also provided few holes, plugged with cotton for air circulation. The bags were transferred to a dark room with 80 per cent relative humidity for spawn run. Spawn run beds were cased with sand, soil and calcium carbonate (1:1:0.18 w/w) after three weeks. The experiment was laid in Non Factorial CRD with four replications.

EXPERIMENT 2

Treatments

T_1-Neemcake 2 per cent

T_2- Neem cake 4 per cent

T_3- Rice bran 2 per cent

T_4- Rice bran 4 per cent

T_5- Ferrous sulphate 5ppm

T_6- Ferrous sulphate 10ppm

T_7- Vermicompost 2 per cent

T_8- Vermicompost 4 per cent

T_9- Control (Without any supplement)

Criteria for selection of the best supplement was the time taken for spawn run, sporophore production, size of sporophore, number of sporophores, total yield and incidence of contaminants.

The best supplement from experiment 2 was taken with best substrate (from experiment 1) and its sterilization procedure for further studies.

3.8.3 Effect of Casing Material

Various combinations of casing material were tried to modify the casing to improve yield of milky mushrooms. The beds were laid using sterilized substrates with the best supplement from the above experiment. The spawned beds were kept for incubation at 80 per cent relative humidity for 21 days. The casing of beds were done using different treatments.

EXPERIMENT 3

Treatments

T_1- Coir Pith + Soil (1:1)

T_2- Vermicompost +soil (1:1)

T_3- Clay +Soil (1:1)

T_4- Control (Sand: Soil: calcium carbonate) (1:1:0.18 w/w)

3.9 DEVELOPMENT OF IMPROVED STRAINS HAVING DESIRABLE CHARACTERS

3.9.1 Irradiation

3.9.1.1 Irradiation with UV Rays

Spore prints of *Calocybe indica* were made on a sterile petri dish. The spores were lifted from dish with a little amount of sterile water and dispersed in sterile distilled water taken in a test tube. Spore suspension was exposed to ultraviolet rays at two distances (5 cm, 10 cm) from source with different time of exposure (10, 15, 20, 25 minutes). The irradiated spore suspension was poured in sterile dish over which potato dextrose agar medium was added. The germinated spores were individually picked and placed on PDA slant to obtain pure cultures. From these pure cultures, mother spawn were prepared on paddy grains. The beds were laid on polyethylene bags using the substrate. Casing of beds were done using sand, soil and calcium carbonate after 21 days. The experiment was laid in CRD (2 x 4 + 1) with three replications

Treatments

T_1- 5cm for 10 minutes

T_2- 5cm for 15 minutes

T_3- 5cm for 20 minutes

T_4- 5cm for 25 minutes

T_5- 10 cm for 10 minutes

T_6- 10 cm for 15 minutes

T_7- 10 cm for 20 minutes

T_8- 10cm for 25minutes

T_9- Control

3.9.1.1.1 Radial Mycelial Growth of Irradiated Cultures of *Calocybe*

The cultures of *Calocybe* irradiated with UV light at two distances and different time of exposure were tested for their growth on the medium. Sterilised, melted and cooled PDA medium was poured in to a sterile petridish. A disc of

5mm cut with the help of a cork borer from the actively growing *Calocybe* cultures (irradiated and control) was placed at the centre of the medium. The nature of growth and radial growth of the mycelium was observed

3.9.1.1.2 Evaluation of Yield Performance of UV Irradiated Cultures

The irradiated cultures were used for spawn preparation and further for laying out beds. The yield performances of irradiated cultures were compared with that of parent culture. The characters evaluated were number of sporophores harvested, time taken for mushroom production, yield and biological efficiency.

3.9.1.1.3 Evaluation of Nutrient Contents

The nutrient contents in the UV irradiated mushroom cultures were evaluated. The characters assessed include moisture content, dry weight, protein content, total soluble solids and fibre content.

3.9.1.1.4 Evaluation of Shelf life of Irradiated Cultures

To assess the effect of irradiation, mushroom of irradiated cultures and control were kept both in room temperature and refrigerated condition. The mushrooms were assayed for their firmness, colour, texture, and decomposition regularly.

3. 9. 1 .2 Irradiation with Gamma (γ) Rays

The cultures of selected isolates of *Calocybe* which showed excellent performance in yield trials were selected for irradiation were grown in test tube. The cultures were subjected to γ radiation on the seventh day after inoculation. This was done at the Radio Tracer Laboratory, Kerala Agricultural University Thrissur. Co 60 was used as the source of γ radiation. Irradiation was done at different levels *viz.*, 0.5KR, 1KR, 1.5KR, 2KR and 2.5 KR. The irradiated cultures were tested for cultural characters including rate of mycelial growth and were later subjected to yield trials.

3.9.1.2.1 Radial Mycelial Growth of γ Irradiated Cultures of *Calocybe*

The cultures irradiated at different levels were tested for the radial mycelial growth on culture medium. Twenty ml of PDA was poured into sterile petridish on which a disc of 5mm cut with the help of a cork borer was taken form the actively growing mycelium of *Calocybe*. The disc was placed at the centre of the medium. The nature of growth and radial mycelial growth was measured after a week. The non-irradiated culture served as control. The experiment was laid out in CRD with three replications.

3.9.1.2.2 Evaluation of Yield Performance of γ Irradiated Cultures

The irradiated cultures were used for spawn preparation and later used for laying out beds. The yield performance of the irradiated cultures at different dose levels were compared with the parent culture. The characters evaluated included number of sporophores, yield, and biological efficiency.

3.9.1.2.3 Evaluation of Nutrient Contents of γ Irradiated Cultures

The study was conducted to evaluate the variation in nutrient content of the irradiated mushroom cultures. The characters evaluated were moisture content, dry weight, protein content, total soluble solids, and fibre content as described in 3.9.

3.9.1.2.4 Evaluation of Shelflife of γ Irradiated Cultures

To assess the effect of irradiation on shelf life of mushroom of irradiated cultures and control were kept at room temperature and refrigerated condition. The mushrooms were assayed for their firmness, colour, texture, and decomposition regularly.

3.9.2 Mating/Crossing of Two Isolates of Dissimilar Characters

3.9.2.1 Multi Spore Crossing

Seven day old cultures of different isolates of milky mushroom were grown on petri dish. Isolates of *Calocybe* were tested for their effect on growth by dual plate method (Utkhede and Rahe, 1983). Potato dextrose agar was

allowed to solidify for one hour in sterilised petri dishes. Then each 3 mm disc of *Calocybe* was placed on the medium 2 cm from the edge of petri dish. Just opposite to that a 3 mm disc of another isolate of the mushroom was placed. Three replications were maintained for each treatment. After the growth of hypae towards each other, development of thick strands of mycelium indicates crossing of two isolates with dissimilar character. In absence of mating the two mycelium either diverges or over grows.

3.9.2.2 Single Spore Isolation

Isolation of single spores was done using serial dilution. Spore print of the mushroom was taken in a sterile dish. The spore mass from spore print was lifted using an inoculation needle and serially diluted in 10 ml sterile distilled water to obtain serial dilution of 10^{-4} ,10^{-3}, 10^{-2}. From these dilutions, 1ml of spore suspension was transferred to each petri dish over which cooled media was added. The petri dishes were incubated for 24 to 48 hours at 28 ± 1°C. For single spore isolation, the germinated single spores were marked with a fine tip marker pen under a dissection microscope. The marked germinated spores were lifted with the help of a fine inoculation needle and transferred to PDA slants. These single spores were then incubated at 28 ± 2 °C for one week. After one week single spore isolates were observed for their growth.

3.9.2.3 Crossing of Single Spores/ Hybridisation

The single spores isolated by the above method were grown in PDA slants. A 3mm disc of one single spore culture of *Calocybe* was placed 2cm away from the edge of a petri dish containing PDA. Another 3mm disc of another single spore culture of *Calocybe* was placed 3.5 cm away from this. Three replications were made for each cross. Growth of the mycelium of each isolates occurs in opposite directions. When the mycelium of each isolate grows and intercept, there was development of thick strand of mycelium which indicates crossing of isolates of dissimilar character.

The thick strand of mycelium developed was transferred to cooked paddy grains for spawn production. After a month this spawn was used for laying out beds. Beds of parents and crosses were compared with others for the number of sporophores harvested, yield, moisture content, fibre content, protein, total soluble solids and enzyme activity.

3.9.2.4 Enzyme Activity

3.9.2.4.1 Phenyl alanine ammonia lyase (PAL)

Activity of PAL was analysed using the procedure described by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenizing one gram mushroom sample in five ml of 0.1 M sodium borate buffer (pH 8.8) containing 0.05 g polyvinyl pyrrolidone using chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant was used for assessing the enzyme activity. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer (pH 8.8), 0.2 ml enzyme extract and 0.1 ml of 12 mM L-phenyl alanine prepared in the same buffer. The blank contained three ml of buffer and 0.2 ml enzyme extract. The reaction mixture and blank were incubated at 40°C for 30 minutes. The reaction was stopped by adding 0.2 ml of 3 N hydrochloric acid after which absorbance was read at 290nm in a spectrophotometer standard curve was prepared using different concentrations of cinnamic acid.

The activity of PAL was expressed as micro gram of cinnamic acid produced per minute per gram on fresh weight basis.

3.9.2.4.2 Peroxidase (PO)

Estimation of peroxidase was done using method described by Srivastava (1987).

Mycelial sample of 200 mg was homogenised in one ml of 0.1 M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone was added. The homogenisation was done at 4 °C using chilled pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 4°C. This supernatant was used as enzyme extract for the assay of peroxidase activity.

The reaction mixture consisting of 1 ml of 0.05 M pyrogallol and 50 µl enzyme extract was taken in both reference and sample cuvettes, mixed and placed in the spectrophotometer and reading adjusted to zero at 420 nm. The enzyme reaction was started by adding 1 ml of one percent hydrogen peroxide in the sample cuvettes and change in absorbance was measured at 30 seconds interval.

3.9.2.4.3 Poly phenol oxidase (PPO)

Poly phenol oxidase was determined as per the procedure of Mayer *et al.* (1965). 200 mg of filter dried mycelium was homogenized in one ml of 0.1 M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone was added. Homogenization was done at 4°C using chilled pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was used as enzyme extract. The reaction mixture contained one ml of 0.1 M sodium phosphate buffer (pH 6.5) and 1 ml of 0.01 M catechol. The cuvette containing the reaction mixture was placed in spectrophotometer and absorbance was set to zero at 495 nm. The reaction was started by adding one ml of catechol to 50 µl of enzyme extract. The change in absorbance was recorded at 495 nm and PPO activity expressed as change in absorbance of reaction mixture per minute per gram on fresh weight basis.

3.10 MOLECULAR CHARACTERISATION

3.10.1 Molecular Characterisation Using Isozyme Analysis

The mycelial disc of mushroom culture *viz.*, parents and hybrid were inoculated into 100ml potato dextrose broth contained in 250 ml conical flask. After two weeks growth in the liquid medium the mycelium was filtered out using Whatman No 1 filter paper. The mycelial mat was removed from the filter paper and rinsed with distilled water. One gram of the sample was ground to a fine powder with liquid nitrogen, with mortar and pestle. Extraction of enzyme was done in 0.05 M Tris buffer in 1:1 proportion. The homogenate was centrifuged for 15 minutes at 4°C and 12000 rpm.

Vertical native polyacrylamide gel electrophoresis was used to separate isozyme using Tris-glycine buffer system. For casting a gel of 20X15cm 12% separating gel mixture (1.5M Tris -2.5 ml, Acrylamide-4ml, TEMED-5ml, Ammonium per sulphide 50 ml Tritonio-100ml and distilled water-3.5 ml) and 4% stacking gel (0.5 M Tris-2.5 ml 10 % Triton-100 ml, Acrylamide-1.3 ml, TEMED-10 ml APS-50 ml, DW-6.1 ml) were used. Clean and dried the glass plates spaces which were assembled properly. Resolved gel solution was degassed using a vacuum pump for 3-5 minutes before adding ammonium per sulphate. The gel (separating) gel was poured in the chamber between the glass plates leaving space for stacking gel. A layer of distilled water was poured on the top of gel and left to set for 60 minutes. Stacking gel after degassing and addition of ammonium per sulphate was poured on to the top of gel after removing the distilled water from top of the gel. Simultaneously the comb was placed in the stacking gel and allowed to settle.

After the stacking gel has polymerised the comb was removed without distorting the shape of the wells. Wells were washed with distilled water using a syringe. The enzyme extract was obtained by grinding 1 g mycelium in 2ml of 50M Tris-chloride at 4°C. One ml of extract with 2ml of bromophenol blue solution was loaded on to the well. The upper and lower tank was filled with electrode buffer and connected upper trough to cathode and lower trough to anode. The sample was run at 50-75 V until it reached the stacking gel. Later the voltage was increased to 100V and run continued until the bromophenol blue reached the bottom of the gel. After the run was complete, gel was removed between the plates and immersed in staining solution. For peroxidase activity gel was incubated in 0.6M sodium acetate buffer (pH 5.4) containing 0.5 per cent O-dianisidine hydrochloride and hydrogen peroxide. It was then incubated for 20 minutes in the dark at 30°C. Poly phenol oxidase activity was analysed by staining the gel in a solution containing 100μ M phosphate buffer (pH 6.0) and DOPA (50 mg) and incubated for 8 hours under light conditions at 30 °C. This was done until the visible bands developed.

3.10.2 RAPD Analysis

3.10.2.1 DNA Extraction and Purification

Genomic DNA from actively growing mycelium was isolated using modified CTAB method. 0.1 gram of dried mycelial sample from each of the parent and hybrid were ground into fine powder using liquid nitrogen. The powdered samples were mixed with 1.5 ml of CTAB extraction buffer (100mM Tris hydrochloride, 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 0.2% mercaptoethanol) and incubated at 65 °C for one hour. After incubation samples were mixed with 400 µl of 24:1 chloroform: isoamyl alcohol and centrifuged at 15,000 rpm for 15 minutes. The aqueous layer was taken and the step was repeated twice. To the aqueous layer two volumes of ethanol and 100 µl sodium acetate was added for precipitation and kept in -20 °C for half an hour. It was then centrifuged.

For DNA purification the removal of proteins and RNA are very essential. For their removal 10 µl of RNAase (10 mg/ml) was added and kept at 40 °C for one hour. Equal volumes of 25:24:1 phenol: Chloroform: isoamyl alcohol (25:24:1) was added mixed and the aqueous layer was collected after centrifugation at 10,000 rpm for 10 minutes. Precipitation was carried out using two volumes of ethanol. The DNA pellets were washed thrice in 70 per cent ethanol, air dried and dissolved in 100 µl of 0.1X TE buffer. The purified DNA was electrophoresed in a 0.7 per cent agarose gel to assess its concentration and integrity. The quantity and purity of the samples were checked using UV/VIS spectrophotometer (GENESYS 3) at OD 260 and OD 280 respectively.

3.10.2.2 PCR Reactions and Gel Analysis

The RAPD analysis was done using eight decamer primers OPA-01, OPA-04, OPP-01, OPP-02, OPP-03, OPP-04, OPP-06, OPP-12, and OPP-16 from Operon Technologies, USA. The PCR amplification was carried out in PTC-150, Thermal cycler, MJ Research. Each reaction mixture of 25µl consisted of 1X PCR

buffer, 2.5 mM Mg Cl2, 1 U Taq DNA Polymerase, 200 µM each of dNTP, 10 picomoles of primer and 100ng of DNA. The PCR conditions were as follows

A preliminary denaturation at 95 °C for 4 minutes, followed by 45 cycles of template denaturation at 94 °C for 1 minute, primer annealing at 36°C for one minute, DNA amplification at 72 °C for two minutes and final primer extension at 72 °C for eight minutes.

The amplification products were electrophoresed on 1.2% agarose gel at 50 volts in 1XTAE buffer. A 100 bp DNA ladder was used as standard marker. The gel was stained with ethidium bromide visualised and photographed using GEL documentation system (BioRad, USA).

Individual bands were scored as 1 or 0 for their presence or absence of RAPD bands. The bivariate data were analysed to generate Jaccard's similarity coefficient. Jaccard's similarity coefficient values for each pair wise comparison between accessions were calculated and similarity coefficient matrix was constructed. The matrix was subjected to unweighted pair group method to generate a dendrogram. All the numerical analysis was conducted using the software NTSYS.

Results

4. RESULTS

4.1 SURVEY

Surveys were conducted in different localities of Thiruvananthapuram, Kollam and Pathanamthitta districts to collect the native flora of *Calocybe* under natural conditions during rainy and post rainy seasons.

4.1.1 Collection of Native Isolates of *Calocybe*

Various isolates of *Calocybe* were obtained from the above mentioned localities. The native strains of *Calocybe* obtained from Thiruvananthapuram district are from Balaramapuram, Chirayankizhu, Nedumangadu, Koliyoor, Vellayani, Pattom and Kattakada. Isolates were also obtained from two locations of Kollam district namely Paravur and Kundara. One sample was obtained from Konni of Pathanamthitta district. The habit of these isolates varied from solitary to gregarious type and they were terrestrial. Organic matter rich soil, coconut tree basin and coirpith were found to be the usual spots for the occurrence of *Calocybe*. The period of occurrence of these mushrooms were from June-October. The features of native isolates of *Calocybe* with their accession numbers from the National Culture Bank, NRCM, Solan are presented in Table 1, Plates 2a, 2b, 2c and 2d.

4.2 IDENTIFICATION OF NATIVE ISOLATES

The accessions obtained were examined for their macroscopic details such as shape, size and colour of basidiocarp. Morphological features of various isolates are given in Table 2. The shape of the pileus of these isolates were convex, off white to creamish white in colour and fleshy in texture. The diameter of the pileus of these isolates range from 5-25 cm. The characters of the stipe were clavate shaped, smooth or scaly surface, centrally attached to the pileus with bulbous base. The length of stipe of each isolate of *Calocybe* ranged from 8-16 cm with a diameter of 2-12 cm.

Table 1 Characteristics of isolates of *Calocybe* under natural conditions

Name of Isolate	Habit	Habitat	Substrate	Place of Collection	Month of occurrence
OE-349	Solitary	Terrestrial	Soil	Balaramapuram	October-2003
OE-350	Solitary	Terrestrial	Organic matter rich soil	Koliyoor	June-2003
OE-351	Solitary	Terrestrial	Coconut tree base	Vellayani	July-2004
OE-352	Solitary	Terrestrial	Basin of coconut tree	Chirayankizhu	July-2003
OE-353	Gregarious	Terrestrial	Coconut tree base	Pattom	July-2003
OE-354	Gregarious	Terrestrial	Coir pith	Kollam	June-2003
OE-355	Solitary	Terrestrial	Coconut tree base	Attingal	October-2003
OE-356	Solitary	Terrestrial	Coconut tree base	Nedumangad	September-2004
OE-357	Gregarious	Terrestrial	Coconut tree base	Kollam	August-2003
OE-358	Solitary	Terrestrial	Coconut stump	Konni, Pathanamthitta	July-2003

Table 2 Morphological features of various isolates of *Calocybe*

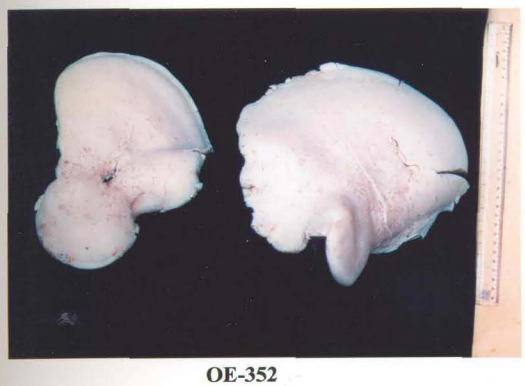
Pileus						Stipe					
Isolate	Shape	Colour	Dia (cm)	Thick (cm)	Texture	Length(cm)	Dia (cm)	Shape	Attachment of pileus	Basal part	Surface
OE-349	Convex	Creamish white	5.0	1.5	Fleshy	12.0	2-4.8	Clavate	Central	Bulbous	Smooth
OE-350	Infundibuliform	Offwhite	18.0	2.5	Fleshy	8-10	5-6	Clavate	Central	Bulbous	Smooth
OE-351	Convex	White	18.0	4.0	Fleshy	13.0	2.5-5	Clavate	Central	Bulbous	Smooth
OE-352	Convex	Offwhite	25.0	4.0	Fleshy	9-10	5-6	Clavate	Central	Bulbous	Supressed Scales
OE-353	Convex	White	12.0	5.0	Fleshy	12-16	8-9	Clavate	Central	Bulbous	Smooth
OE-354	Convex	Offwhite	8.0	2.0	Fleshy	9.5	2.5-6	Clavate	Central	Bulbous	Smooth
OE-355	Convex	Creamish white	18.0	6.0	Fleshy	11.5	9.5-12	Clavate	Central	Bulbous	Smooth
OE-356	Convex	Offwhite	20.0	4.0	Fleshy	7-9	5-6	Clavate	Central	Bulbous	Scaly
OE-357	Convex	White	10.0	3.5	Fleshy	15.0	3-4.5	Clavate	Central	Bulbous	Smooth
OE-358	Convex	Offwhite	12.0	3.0	Fleshy	16.0	4.-8	Clavate	Central	Bulbous	Smooth

OE-349

OE-350

Plate 2a Natural collections of *Calocybe*

OE-351



OE-352

OE-353

Plate 2b Natural collections of *Calocybe*

OE-354

OE-355

Plate 2c Natural collections of *Calocybe*

OE-356

OE-357

OE-358

Plate 2d Natural collections of *Calocybe*

The characters of gills namely arrangement, texture, margin, gill/cm, spore print, spore colour and spore shape are given in Table 3.

4.2.1 Methodology for Identification

4.2.1.1 Microchemical Reagents

These reagents enabled to identify mushrooms.

4.2.1.1.1 Melzers Reagent

The treatment of spores taken from spore print with Melzers reagent showed nonamyloid / in amyloid reaction with yellow coloured spores.

4.2.1.1.2 Cotton blue Stain

The cotton blue treatment of spores gave cyanophilic reaction by changing the spore wall colour to blue or dark blue.

4.2.2 Screening of Isolates for Organoleptic Characters

The native isolates screened for their characters like colour, appearance, texture and flavour on the basis of score card were subjected to Kruskal Wallis statistical test and ranked. The details are presented in Table 4. Among the different isolates, OE-357 had maximum ranking for colour appearance and flavour followed by the isolate, OE-349 which also had better texture. These two isolates were most preferred by the judges than the other isolates. The ranking of isolates based on the colour ranged from 17.8-86.75, where as for appearance it was 23.2- 84.75. The ranking of isolates based on the texture and flavour were 24.55-62.30 and 34.90-63.15 respectively (Fig. 1).

4.3 ISOLATION AND PURIFICATION OF CULTURE

The tissue isolation of the isolates was done as per the standard method described under 3.3.1 and the cultures were maintained on PDA slants by periodical subculturing.

Table 3 Characters of different native isolates

	Gills				Spore				
Isolate	Arrangement	Texture	Margin	Gills/cm	Spore Colour	Spore print	Shape	Cotton blue reaction	Melzers reaction
OE-349	Free	Opaque	Smooth	14	Hyaline	White	Globose	Cyanophilic	Nonamyloid
OE-350	Free	Brittle	Smooth	12	Hyaline	White	Ovate	Cyanophilic	Nonamyloid
OE-351	Free	Brittle	Smooth	14	Hyaline	White	Globose	Cyanophilic	Nonamyloid
OE-352	Free	Opaque	Smooth	12	Hyaline	White	Ovate	Cyanophilic	Nonamyloid
OE-353	Free	Soft	Smooth	12	Hyaline	White	Ellipsoidal	Cyanophilic	Nonamyloid
OE-354	Free	Soft	Smooth	11	Hyaline	White	Globose	Cyanophilic	Nonamyloid
OE-355	Free	Opaque	Smooth	10	Hyaline	White	Ovate	Cyanophilic	Nonamyloid
OE-356	Free	Soft	Smooth	12	Hyaline	White	Ovate	Cyanophilic	Nonamyloid
OE-357	Free	Opaque	Smooth	14	Hyaline	White	Ovate	Cyanophilic	Nonamyloid
OE-358	Free	Soft	Smooth	12	Hyaline	White	Ovate	Cyanophilic	Nonamyloid

Table 4 Screening of isolates for organoleptic characters

Sl. No	Isolates	Colour*	Appearance*	Texture*	Flavour*
1	**OE- 349**	**72.40**	**79.25**	**62.30**	**58.50**
2	OE-350	47.80	41.80	62.30	46.25
3	OE-351	43.70	45.75	53.20	58.5
4	OE-352	23.70	31.10	24.55	48.85
5	OE-353	56.00	69.80	50.20	55.55
6	OE-354	44.85	33.90	45.65	55.55
7	OE-355	51.90	49.70	45.65	42.15
8	OE-356	17.80	23.20	50.2	34.90
9	**OE-357**	**86.75**	**84.75**	57.75	**63.15**
10	OE-358	60.10	45.75	53.20	41.6
CD (0.05)		25.429	25.429	25.429	25.429

*Average of 10 ranks

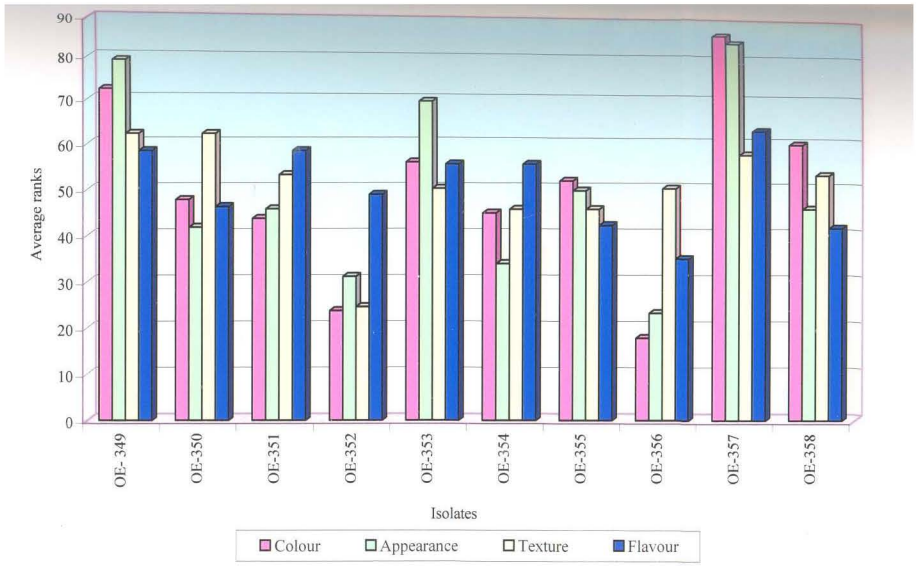


Fig. 1 Comparative organoleptic characters of native isolates

4.4 SCREENING OF MUSHROOM ACCESSION UNDER *IN VITRO* CONDITIONS

4.4.1 Nature and Growth of *Calocybe* Isolates

Growth of *Calocybe* isolates in petridish was recorded seven days after inoculation. The study indicated that isolates OE-358, OE-353, OE-352 and OE-354 were on par, recording a radial growth of 4.03, 4.13, 4.23 and 4.27 cm respectively. These isolates were followed by OE-356 and OE-351 having 4.36 cm and 4.73 cm growth. The isolates OE-351, OE-349, OE-355 were on par with 4.73, 4.80 and 4.83 cm of growth respectively. Among the ten isolates OE-357 and OE-350 were on par which gave better growth of 5.00 and 5.30 cm respectively (Table 5 and Fig. 2).

Growth of *Calocybe* isolates on PDA medium indicated the attainment of full growth in fourteen days. The isolates OE-349, OE-357, OE-350, OE-355 and OE-356 were on par and completed 9.00-8.73 cm growth in petridish after 14 days. The isolates OE-351 and OE-352 were on par with OE-356 having growth of 8.43 cm, 8.23 cm respectively. These isolates were significantly different from the isolates OE-358, OE-353, OE-354 having respectively 7.37, 7.20 and 7.16 cm of growth. So the isolates OE-349, OE-357, OE-350, OE-355 and OE-356 were the best (Table 5 and Plate 3).

Nature of mycelial growth in PDA slants indicated that isolates OE-349, OE-351, OE-355 and OE-357 were fluffy in nature when compared to the others. Isolates OE-350, OE-352, OE-353, OE-354 and OE-358 had medium fluffy growth, whereas the isolate OE-356 had feable growth of mycelium (Table 5 and Plate 4).

Table 5 Growth of *Calocybe* isolates in petridishes (PDA)

Sl. No	Isolate	Growth after 7 days (cm)*	Growth after 14 days (cm)*	Nature of mycelial growth (in PDA slants)
1	OE-349	4.80	9.00	++++
2	OE-350	5.33	8.97	+++
3	OE-351	4.73	8.43	++++
4	OE-352	4.23	8.23	+++
5	OE-353	4.13	7.20	+++
6	OE-354	4.27	7.17	+++
7	OE-355	4.83	8.93	++++
8	OE-356	4.37	8.73	++
9	OE-357	5.07	9.00	++++
10	OE-358	4.03	7.37	+++
	CD (0.05)	0.295	0.547	

*Average of three replications

++++ - Fluffy, +++- Medium, ++ - Feable

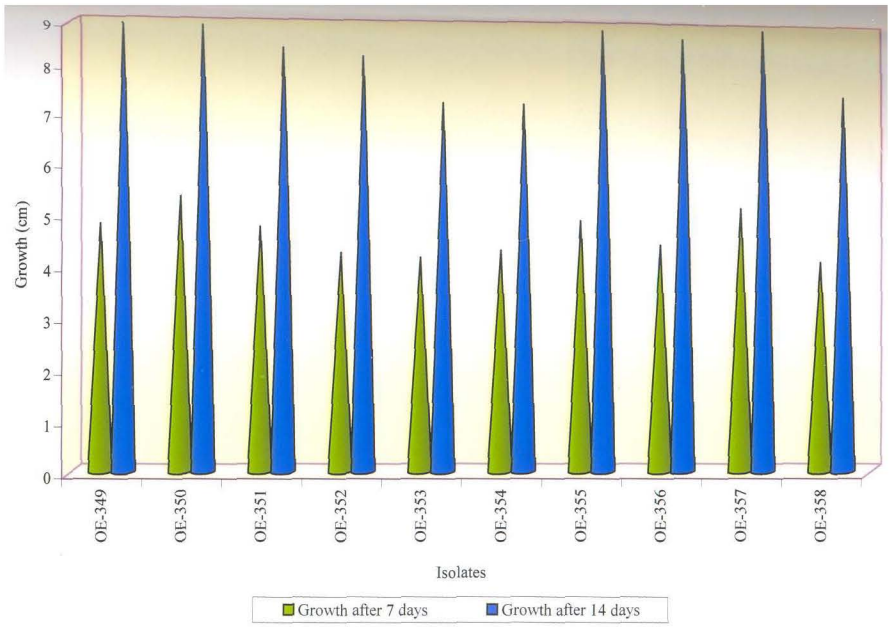


Fig. 2 Growth of *Calocybe* isolates in petridishes (PDA)

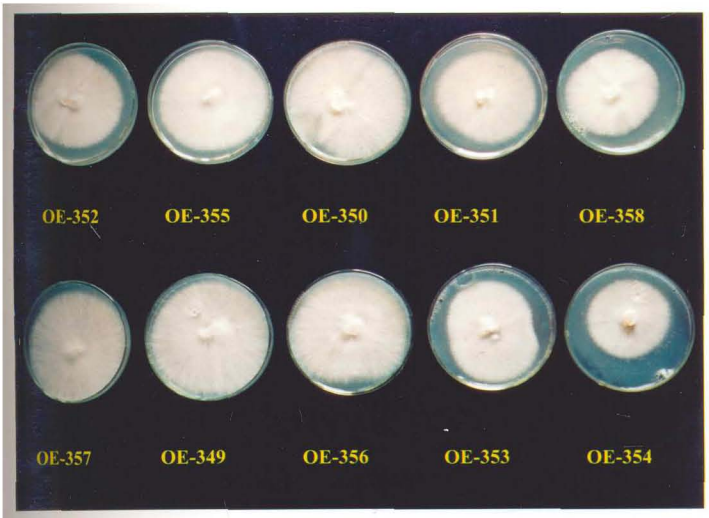


Plate 3 Growth of native isolates 14 days after inoculation

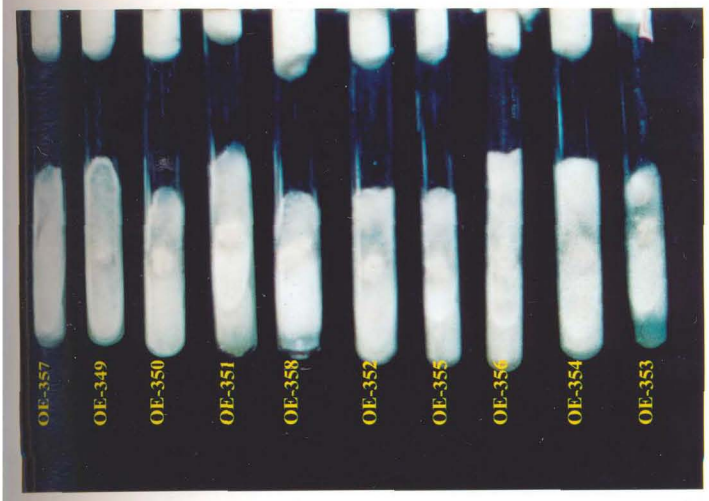


Plate 4 Growth of natural collections in PDA slants

4.5 SCREENING OF MUSHROOM ACCESSIONS FOR COMMERCIAL CULTIVATION

4.5.1 Preparation of Spawn

Spawn was prepared in paddy grains as described under 3.5.1.

4.5.1.1 Nature of Growth and Period for Spawn Run

Among the different isolates, OE-349, OE-351, OE-355 and OE-357 had better fluffy growth of the mycelium in the grains when compared to other isolates. Lesser growth was observed in isolates OE-350, OE-353, OE-356 and OE-358. Least growth was observed in isolates OE-352 and OE-354 (Table 6).

The time taken for spawn run in the case of mother spawn varied widely among different native isolates. Isolate OE-355 took the minimum period for spawn run *i.e.*, 18.33 days. The isolates of OE-350, OE-352, OE-349, OE-357 and OE-351 were on par which required 19.67 – 20.67 days for complete spawn run. Isolates OE-351, OE-358 and OE-354 took 20.67, 21.67 and 22.33 days respectively. The isolate OE-356 took 23.67 days. The maximum number of days for spawn run was observed in the isolate OE-353 with 26.67 days (Table 6 and Plate 5).

The time taken for the growth of other spawn of the ten isolates varied from 11.33 – 14.33 days. The isolates OE-349, OE-357, OE-351, OE-354, OE-355 and OE-352 took 11-13 days. The isolates OE-350, OE-356, OE-353 and OE-358 took 13.33, 13.67, 13.67 and 14.33 days respectively (Table 6 and Fig. 3).

4.5.2 Screening Trials

Cultivation of different isolates was done using chemically sterilized paddy straw (75 ppm carbendazim and 500ppm formalin) on polythene bags of 60 x 45 cm with layer spawning as explained under 3.5.2. For each isolate time taken for initial appearance, days for harvest and total days taken for harvest from bed preparation were observed (Table 7).

Table 6 Nature of mycelial growth of various isolates

Sl No	Isolate	Growth on grains	No of days taken for growth of mother spawn*	No of days taken for growth of spawn*
1	OE-349	++++	20.33	11.33
2	OE-350	+++	19.67	13.33
3	OE-351	++++	20.67	11.33
4	OE-352	++	20.33	13.00
5	OE-353	+++	26.67	13.67
6	OE-354	++	22.33	12.33
7	OE-355	++++	18.33	12.67
8	OE-356	+++	23.67	13.67
9	OE-357	++++	20.67	11.33
10	OE-358	+++	21.67	14.33
	CD (0.05)		2.306	1.866

*Average of three replications

++++ - Fluffy, +++- Medium, ++ - Feable

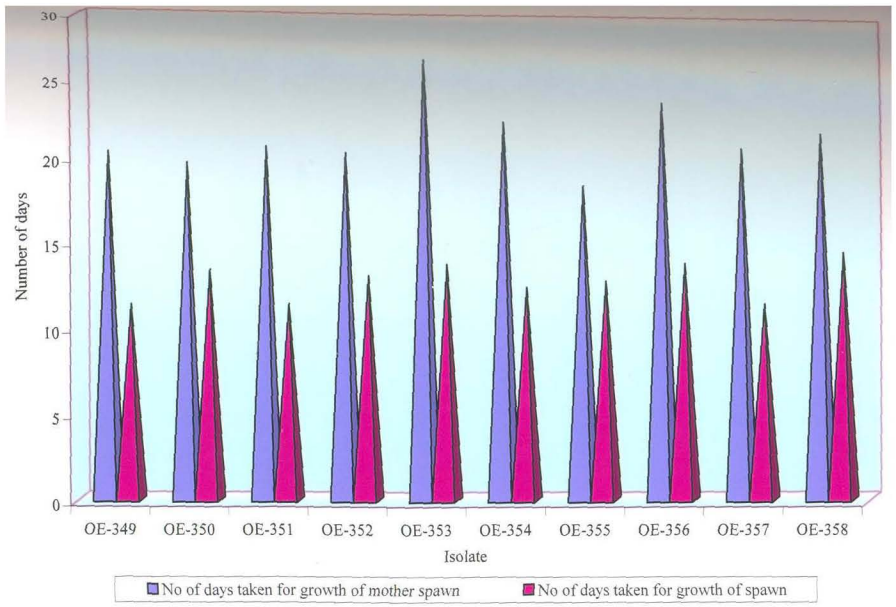


Fig. 3 Duration for spawn growth

1. OE-355
2. OE-349
3. OE-350
4. OE-351
5. OE-352
6. OE-357
7. OE-358
8. OE-354
9. OE-356
10. OE-353

Plate 5 Duration for spawn growth of native isolates

4.5.2.1 Comparative Characters of Isolates on Beds

The native isolates of *Calocybe* showed wide variation in the number of days taken for the primordial initiation. The days taken for initial appearance varied from 8-20 days among the native strains. The isolate OE-349 took only eight days for initial appearance and it was on par with two other isolates OE-358 and OE-351 with 10 and 10.33 days respectively. The isolates OE-357, OE-353 and OE-352 which were on par have taken slightly more number of days when compared to the above mentioned ones. The days taken for initial appearance in isolates OE-350, OE-354 and OE-355 were 12.67, 13.67, and 13.33 days respectively. There was significant difference between the isolates OE-356 and OE-355. The isolate OE-356 took a maximum period of 20.00 days for initial appearance of primordia. The days taken for initial appearance is to be minimum for early harvest which may be a preferred criteria for the selection of a strain. Among the ten isolates OE-349 was the best with minimum period for production of initials when compared to the isolate OE-356 which took the maximum number of days (Fig. 4).

The harvesting period also varied widely among the different strains of *Calocybe*. The isolate OE-349 took minimum period for harvest with 16.67 days. The isolates OE-351, OE-358, OE-353, OE-350, OE-354, OE-352 and OE-355 required a period ranging from 20.00-22.66days whereas the isolate OE-357 took 24.33 days. The maximum period for harvest was noticed in the isolate OE-356. Since the minimum harvesting period was preferred, isolate OE-349 was superior than OE-357 (Table 7).

The maturity period did not vary significantly between the different isolates. The maturity period of the various isolates ranged from 8.67-12.67 days with maximum of OE-357 and minimum with OE -349, OE-350, and OE-355 (Table 7).

The days taken for harvest from bed preparation ranged from 37.66 – 50.00 days. The minimum period was noticed in the isolate OE-349 and

Table 7 Comparative characters of isolates on beds

Isolate	Days taken for appearance of initial (after casing)	Days taken for harvest	Maturity period	Total no of days taken for harvest from bed preparation
OE-349	**8.33**	**16.67**	**8.67**	**37.66**
OE-350	12.67	21.33	8.67	42.33
OE-351	10.33	20.00	9.67	41.33
OE-352	12.33	22.33	9.67	43.33
OE-353	11.67	21.00	9.33	42.00
OE-354	13.67	22.33	9.00	43.33
OE-355	13.33	22.66	8.67	44.00
OE-356	20.00	29.33	9.33	50.00
OE-357	11.67	24.33	12.67	45.33
OE-358	10.00	20.33	10.33	41.33
CD (0.05)	1.991	3.125		3.109

Average of three replications

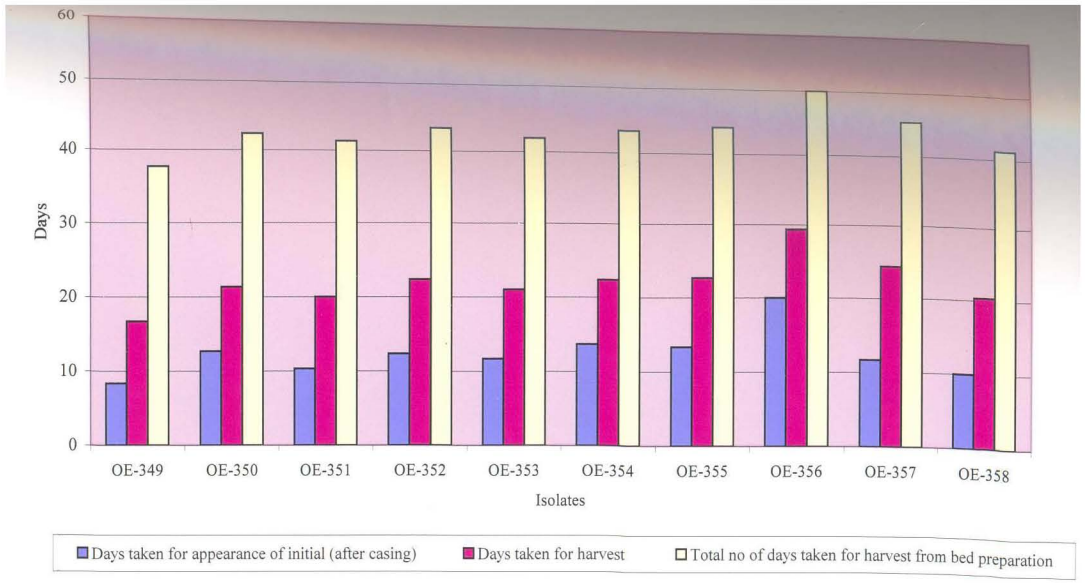


Fig. 4 Comparative characters of *Calocybe* isolates on beds

maximum with the isolate OE-356. The other isolates had taken 41.33-45.33 days for harvest (Table 7 and Fig. 4).

4.5.2.2 Comparative Yield Performance of Native Isolates

Performance of isolates was tested with the observations on mortality percentage, number of sporophores harvested from a bed, yield and biological efficiency (Table 8).

The mortality percentage of various isolates were tested on beds which showed wide variation. The isolate OE-355 produced only one sporophore initial, hence had no mortality. The lowest mortality percentage was observed in the isolate OE-349 (55.55%) and highest in isolate OE-356. The isolates OE-357, OE-350, OE-354, OE-351, OE-352 and OE-353 had mortality percentage of 70.52, 72.29, 74.39, 74.58, 74.59 and 76.59 per cent respectively, which were on par. The isolate OE-349 was recorded as superior isolate due to the low mortality percentage when compared to other isolates (Fig. 5).

Maximum number of sporophores was obtained from the isolate OE-349 (11.67). The isolate OE-355 produced only one sporophore per bed which was on par with the isolates OE-353, OE-354 and OE-356which had produced 2.33, 3.67, and 4.33 sporophores respectively. The isolates OE-352, OE-351, OE-350, and OE-358 were considered superior to others as the number of sporophore produced were 6.33, 6.33, 7.33 and 7.67respectively. Isolate OE-358 which was on par with OE-357 produced 9.33 sporophores. The isolate OE-357 was not significantly different from OE-349. The isolate OE-349 was considered superior and OE-355 as inferior.

Yield

The yield obtained was estimated on g/kg substrate of different native strains. The study indicated that the isolates OE-355, OE-354, OE-356 and OE-353 were on par producing yield of 51.67 g, 61.67g and

Table 8 Comparative yield performance of natives isolates

Isolate	Mortality percentage*	No of sporophores harvested from a bed*	Yield (g/ kg substrate)*	Biological efficiency* (%)
OE-349	**55.55 (7.45)**	**11.67**	**368.33**	**36.83**
OE-350	72.29(8.50)	7.33	318.33	31.83
OE-351	74.58(8.64)	6.33	273.33	27.36
OE-352	74.59(8.64)	6.33	166.67	16.67
OE-353	76.59(8.75)	2.33	126.67	12.67
OE-354	74.39(8.63)	3.67	51.67	5.17
OE-355	0(0)	1.00	51.67	5.17
OE-356	90.85(9. 53)	4.33	61.67	6.17
OE-357	70.52(8. 40)	9.33	341.67	34.17
OE-358	80.27(8.96)	7.67	256.67	25.67
CD (0.05)	0.661	3.532	100.811	10.081

Figures in parentheses are $\sqrt{x+1}$ transformed values

*Average of three replications

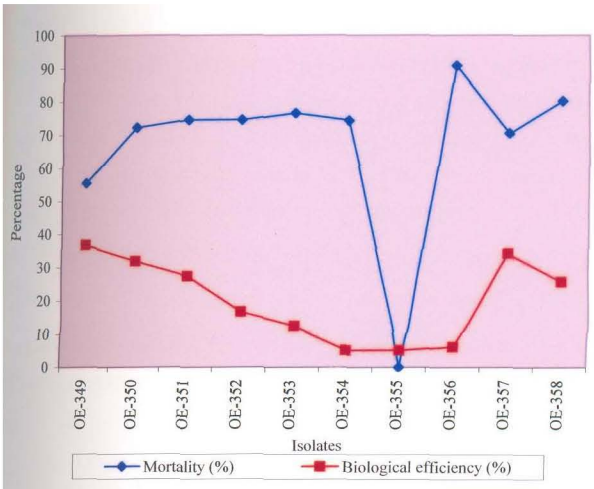


Fig. 5 Yield potential of native isolates

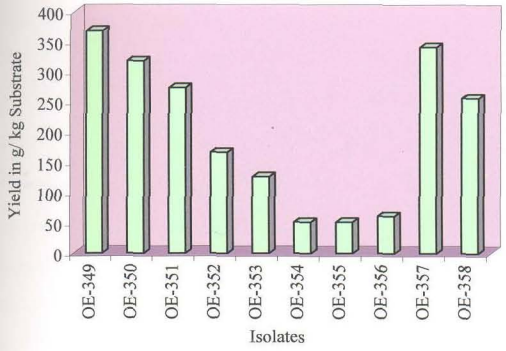


Fig. 6 Comparative yield performance of native isolates

126.67 g respectively. The isolate OE-352 which produced 166.67 g was on par with OE-353 and also with OE-358 having yield of 256.67 g. The isolate OE-351, OE-350 and OE-357 were better than the other isolates producing 273.33g, 318.33g and 341.67 g respectively. The isolate OE-349 proved to be the best one producing maximum yield of 368.33 g compared to the other isolates. The biological efficiency (BE) of each isolate ranged from 5.17 – 36.83 per cent with maximum in OE-349 and minimum in OE-354 and OE-355. In the case of native isolates BE ranged from 6.17 - 34.17 per cent (Table 8, Fig. 6, Plates 6a, 6b and 6c).

4.5.2.3 Comparative Morphology of Native Isolates on Beds

The characters of stipe, pileus and gills were studied. The stipe length of various isolates varied widely. It was observed that the stipe length of various isolates ranged from 5.00 – 10.83cm. The maximum length of 10.83 cm was observed on isolate OE-355 and minimum on OE-354. The isolate OE-358, OE-349, OE-350, OE-351, OE-357 and OE-352 were on par having 5.50 cm, 5.66 cm, 5.83 cm, 6.20 cm, 6.23 cm, and 6.83 cm respectively. The isolate OE-353 was significantly different from the above isolates but was on par with isolates OE-356 and OE-355 with stipe length of 10.83 cm, 8.33 cm, and 9.33 cm, respectively (Table 9).

The diameter of the stipe varied from 4.40 – 8.93 cm with maximum diameter in isolate OE-351 and minimum in OE-352 (Fig.7).

The diameter of the pileus of different native strains did not vary widely but minimum diameter of pileus was observed in OE-354 with 5.50 cm and maximum pileus diameter for OE-349 with 9.73 cm. The thickness of the pileus did not vary much. But thickness varied from 1.06 – 1.63 cm (Fig.7).

Maximum number of gill/cm was observed in the case of isolate OE-355 (17.33) which was significantly different from isolate OE-354

Table 9 Comparative morphology of native isolates

Sl. No	Name of isolate	Stipe*		Pileus*		Gills/cm*	Average weight of fruiting body (g)
		Length (cm)	Diameter (cm)	Diameter (cm)	Thickness (cm)		
1	OE- 349	5.66	7.73	9.73	1.16	9.00	70.00
2	OE-350	5.83	6.66	9.33	1.06	8.67	56.67
3	OE-351	6.20	8.93	8.07	1.20	10.67	58.33
4	OE-352	6.83	4.40	6.90	1.40	10.33	15.00
5	OE-353	8.83	6.10	7.13	1.63	10.33	63.33
6	OE-354	5.00	4.53	5.50	1.27	11.33	10.00
7	OE-355	10.83	6.43	7.57	1.27	17.33	51.67
8	OE-356	9.33	6.66	8.77	1.20	10.33	61.67
9	OE-357	6.23	5.70	7.63	1.67	8.67	35.00
10	OE-358	5.50	6.00	7.37	1.27	8.67	31.67
	CD (0.05)	1.925	1.665	1.628		3.491	35.113

*Average of three replications

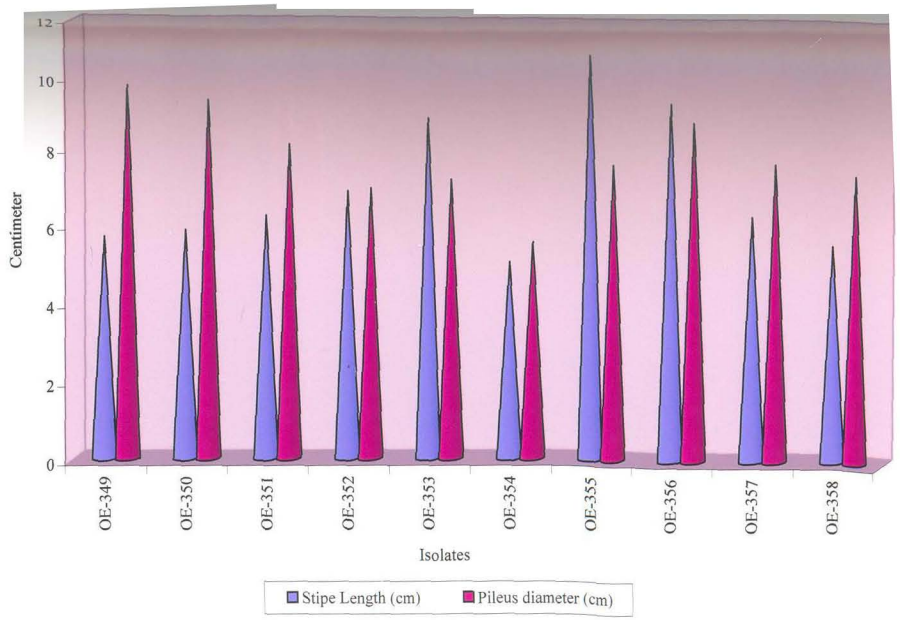


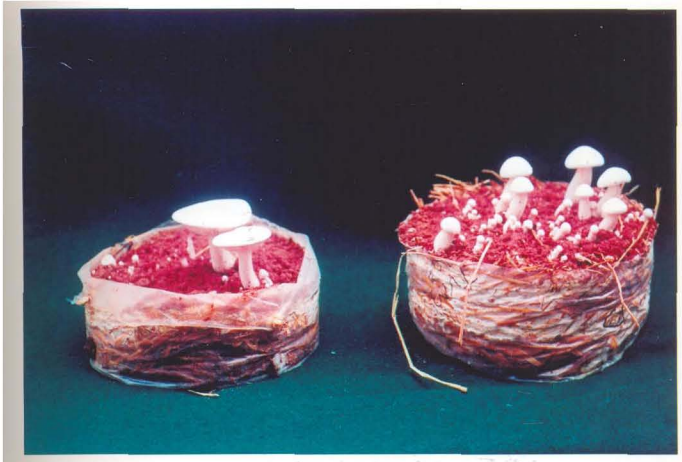
Fig. 7 Comparative morphology of native *isolates*

OE-349

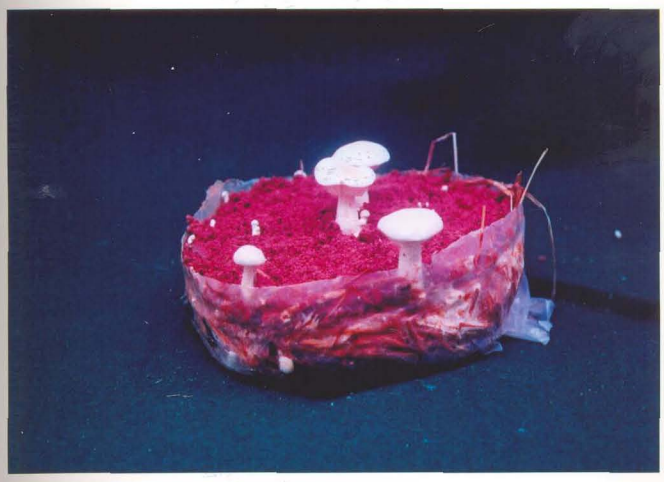
OE-350

OE-351

Plate 6a Natural isolates on artificial cultivation

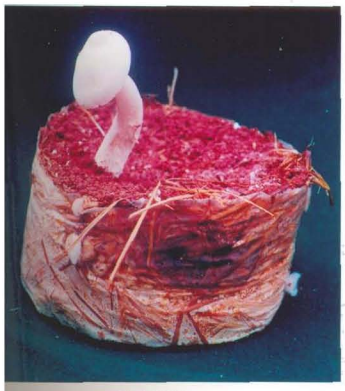


OE-353

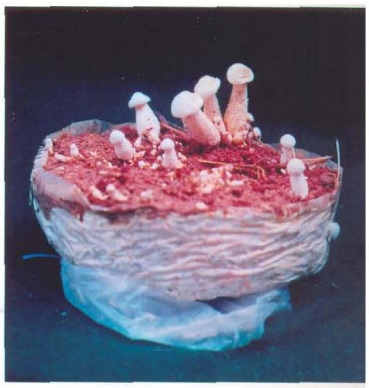


OE-354

Plate 6b Natural isolates on artificial cultivation



OE-355



OE-356

OE-357 OE-358 OE-352

Plate 6c Natural isolates on artificial cultivation

having 11.33 gill/cm. The gills/cm of the other isolates were on par ranging from 8.67 – 10.67.

The average weight of single fruiting body of native isolates on beds was also noted. Lowest weight of fruiting body was noticed in the isolate OE-354 (10.00) which was on par with average fruiting body weight of the isolate OE-352, OE-358 and OE-357. The average weight of fruiting body of the native isolates ranged from 10-70 g. The maximum weight of 70 g was recorded in the isolate OE-349 and minimum in OE-354 *i.e.*, 10 g (Table 9).

4.5.3 Screening for Post harvest Quality

The study conducted to determine the shelf life of native isolates of *Calocybe* indicated that mushrooms when stored in polypropylene covers without perforations had better keeping quality than those kept in polypropylene covers with perforations.

The mushrooms of different isolates when stored at room temperature did not show much variation. Maximum shelf life was observed in the case of the isolate OE-349 (6.67 days) and minimum in OE-354. The shelf life of other isolates ranged from 3.67 – 5.33 days.

The different native isolates had a better shelf life when stored in polypropylenes covers without perforation in a refrigerator. The isolates OE-349 and OE-357 had shelf life of 24.67 and 22.67 days. Minimum period of storage under refrigerated condition was observed in the isolate OE-358, which was on par with isolates OE-354, OE-355 and OE-356 (12.67, 13.3, 15.3, and 15.67 days respectively). The other isolates had a shelf life ranging from 17.33 – 21.33 days (Table 10 and Fig. 8).

4.6 PROXIMATE CONSTITUENTS

4.6.1 Moisture Content

The moisture content of various native isolates ranged between 80.92 and 89.64 per cent. The isolate OE-355 had minimum moisture content of

Table 10 Shelf life of mushrooms after harvest

Sl. No	Name of isolate*	Keeping quality (days)*	
		Room temperature	Refrigerator
1	OE- 349	6.67	**24.67**
2	OE-350	5.00	17.33
3	OE-351	5.33	19.33
4	OE-352	3.67	21.33
5	OE-353	3.67	17.67
6	OE-354	3.00	13.33
7	OE-355	3.67	15.33
8	OE-356	4.33	15.67
9	OE-357	5.00	22.67
10	OE-358	3.00	12.67
	CD (0.05)	1.703	3.744

*Average of three replications

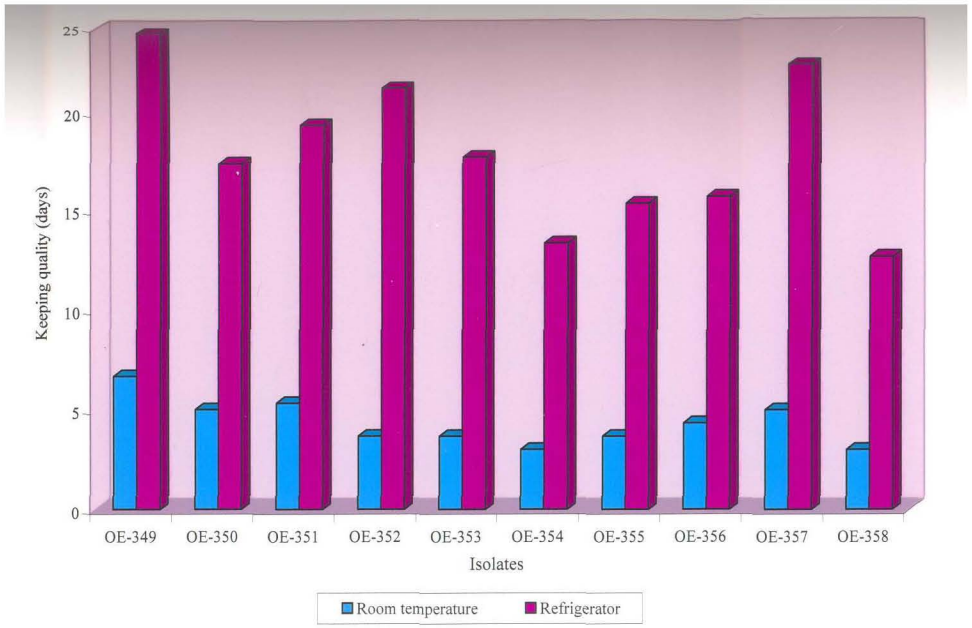


Fig. 8 Shelf life of sporocarps of native isolates

80.92 per cent which was on par with the isolates OE-356 and OE-352 having 81.36 per cent and 81.44 per cent. The isolate OE-352 was on par with the OE-354 which was significantly different from the other isolates. The isolates OE-354, OE-350, OE-357 and OE-353 were significantly different with moisture content of 81.89, 85.48, 85.79, and 87.31 per cent respectively. The moisture content of isolates OE-349 and OE-358 which were on par were 88.22 and 88.59 per cent respectively. The maximum moisture content was noticed in the isolate OE-351 *i.e.*, 89.64 per cent.

The dry weight of various native isolates indicated that the highest dry weight was detected in the isolate OE-355 with 19.08 per cent and lowest dry weight was observed in the isolate OE-351 with 10.36 per cent. The dry weight of the other isolates ranged from 11.40 – 18.57 per cent. The isolate OE-352 was considered superior to OE-355 due to the high dry weight content (Table 11 and Fig. 9).

4.6.2 Total Soluble Solids

There was significant difference between the native isolates of *Calocybe* in total soluble solids. The total soluble solids concentration ranged between 5 and 7 brix with maximum of 7 brix shown by OE-352 and minimum of 5 brix by various isolates (Table 11).

4.6.3 Fibre Content

The lowest fibre content was observed in the isolate OE-349 (16.02 %) and highest in OE-356 (24.41 %). The fibre content of the other isolates ranged from 16.67 to 23.05 per cent. The isolates OE-349, OE-350, OE-353, OE-351 and OE-357 were on par having 16.02, 16.67, 17.05, 17.40 and 17.57 per cent fibre content respectively. The isolate OE-354 and OE-352 were on par with OE-357 with 19.93, 20.49 per cent fibre. The isolate OE-355 was on par with OE-356 with 23.05 and 24.41 per cent fibre content respectively (Table 11 and Fig. 9).

Table 11 Nutrient content of various native isolates of *Calocybe*

Sl. No	Name of isolate	Moisture content (%)*	Dry wt (%)*	Total soluble solids (Brix)	Fibre content (%)*	Protein (µg/g)	Total carbohydrate (µg/g)*
1	OE- 349	88.22	11.77	5	16.02	42	15.67
2	OE-350	85.48	14.52	5	16.67	28	11.67
3	OE-351	**89.64**	10.36	6	17.40	30	18.33
4	OE-352	81.44	18.57	7	20.49	34	13.33
5	OE-353	87.31	12.69	5	17.05	34	20.33
6	OE-354	81.89	18.04	5	19.93	28	20.67
7	OE-355	80.92	**19.08**	5	23.05	32	10.33
8	OE-356	81.36	18.64	6	**24.41**	26	8.33
9	OE-357	85.79	14.21	5	17.57	28	15
10	OE-358	88.59	11.40	6	22.37	30	17.67
	CD (0.05)	0.661	0.642		2.942		5.439

* Average of three replications

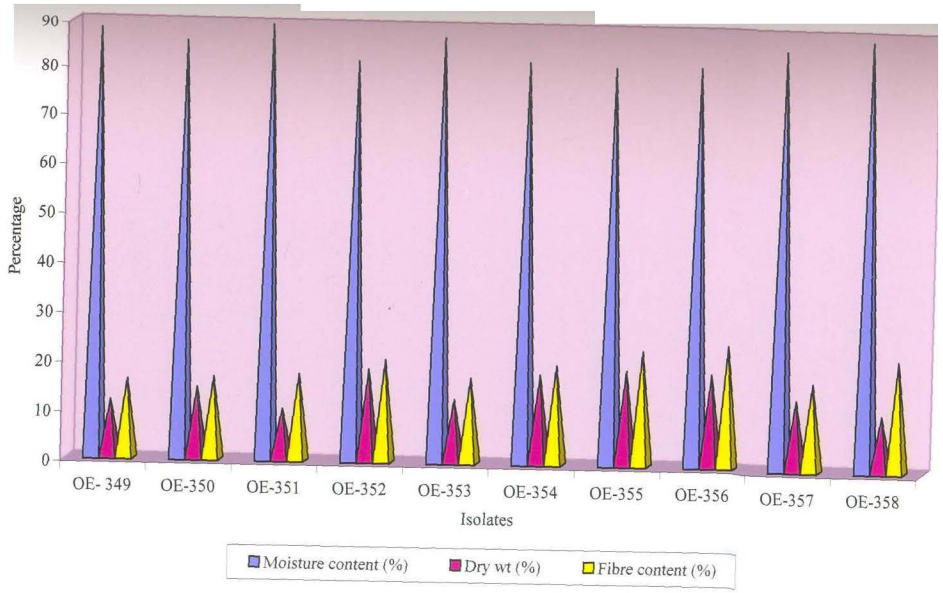


Fig. 9 Proximate constituents of various isolates

4.6.4 Protein

The protein content of the native isolates of *Calocybe* was determined using Bradford method *i.e.*, by taking spectrophotometer readings at 595 nm. It was observed that OE-349 had the maximum protein content of 42 µg of protein/ g of sample. Lowest protein content of 26 µg was observed in the case of OE-356. The isolates OE-352 and OE-353 had 34 µg/g of protein, whereas isolate OE-355 had 32 µg/g of protein. 30µg/g of protein was detected in OE-351, OE-358 and 28 µg on OE-357, OE-350 and OE-354 and 26 µg on OE-356 (Table 11 and Fig.10).

4.6.5 Total Carbohydrates

Total carbohydrate was estimated using anthrone reagent and development of colour read at 630 nm. The lowest carbohydrate content was noticed in isolate OE-356 (8.33 µg/g), and highest in OE-354 (20.67 µg/g). The details on the quantity of carbohydrates are given in Table 11 and Fig.10.

4.7 CULTURAL STUDIES

4.7.1 Evaluation of Growth of *Calocybe* sp. in Different Culture Media

4.7.1.1 Nature and Radial Growth of Calocybe sp.

Five different solid culture media namely potato dextrose agar, malt extract agar, oat meal agar, coconut milk agar and jackfruit seed kernel agar were tested for their efficacy in supporting the radial mycelial growth of *Calocybe* isolate (OE-349). The results showed that the media significantly differed in influencing mycelial growth of *Calocybe* isolate (Table 12).

The nature of mycelial growth of *Calocybe* was very much fluffy in oat meal agar medium followed by potato dextrose agar medium with fluffy growth. In jack fruit seed kernel agar and coconut milk medium, the nature of growth was medium type. Malt extract agar media was inferior in supporting the growth as, it was feable in nature (Plate 7).

Table 12 Growth of *Calocybe* culture (OE-349) in different solid media

Sl. No	Media	Nature of mycelial growth	Growth (cm) after*		No of days taken for full growth*
			7 days	14 days	
1	**Potato dextrose agar**	++++	**6.17**	**8.97**	**14.25**
2	Malt extract agar	++	5.37	8.35	17.49
3	Oat meal agar	+++++	5.27	8.17	17.48
4	**Jack fruit seed kernel agar**	+++	**6.15**	**8.94**	**14.00**
5	Coconut milk agar	+++	5.52	8.52	16.75
	CD (0.05)		0.085	0.030	0.112

*Average of four replications

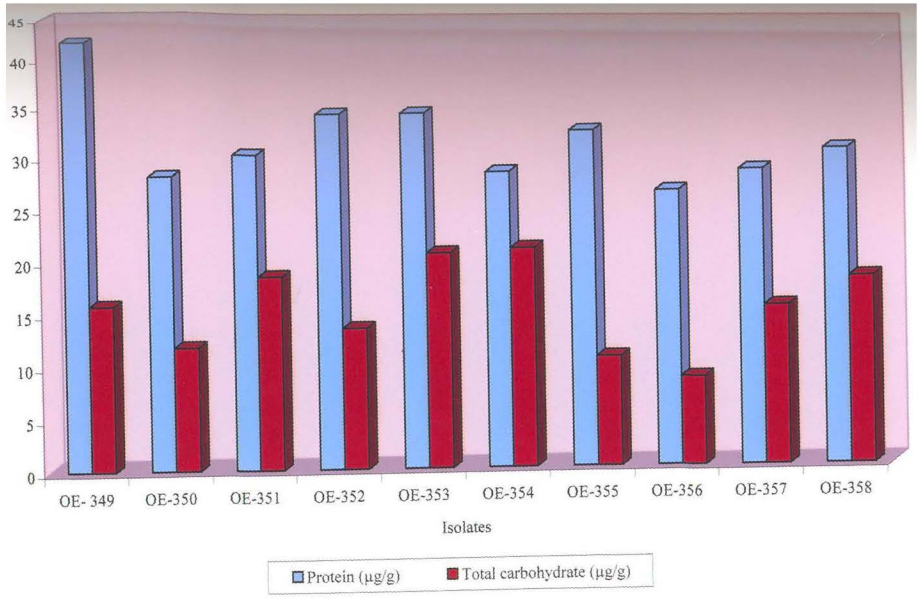
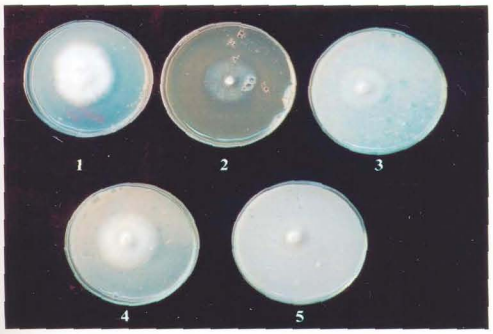
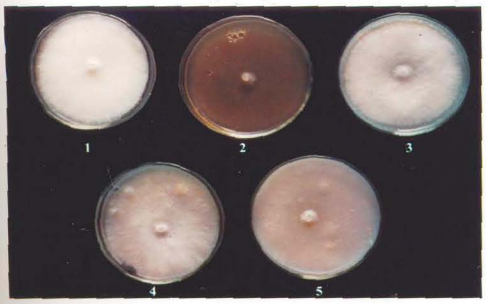


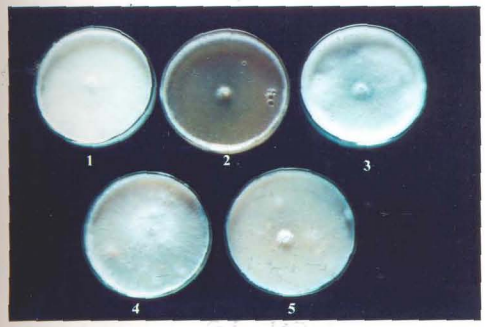
Fig. 10 Protein and carbohydrate content of native isolates



Growth after seven days



Growth after 14 days



Nature of growth

1. Potato dextrose agar
2. Malt extract agar
3. Oat meal agar
4. Jackfruit seed kernel agar
5. Coconut milk agar

Plate 7 Growth of *Calocybe* in different solid media

Radial growth of *Calocybe* culture after seven days indicated that potato dextrose agar and jackfruit seed kernel agar media were considered superior producing 6.17cm and 6.15 cm of radial growth. The other media were on par having a radial growth of 5.27 – 5.52 cm (Plate 7).

The growth of *Calocybe* isolate on petridish was completed in fortnight period with slight variation. The isolate completed the full growth in potato dextrose agar medium and jackfruit seed kernel agar medium in two weeks. In coconut milk agar, it could complete only 8.52 cm growth in two weeks time and took 16.75 days for complete growth. Oat meal agar and malt extract agar took more time for complete growth *i.e.*, 17.48, and 17.49 days respectively. In these media the isolate could grow only 8.17 cm and 8.35 cm in two weeks time (Fig.11 and Plate 7).

4.7.2 Growth of *Calocybe* in Shake and Static Culture

Biomass production of the selected isolate of *Calocybe* was estimated in liquid broths *i.e.*, potato dextrose broth, malt extract broth, oatmeal broth, jackfruit seed kernel broth and coconut milk broth both under shake and static conditions (Table 13).

The results of the study showed that static culture produced comparatively higher biomass than shake culture with an exception in the case of malt extract broth.

The studies conducted on shake culture revealed jackfruit seed kernel broth as superior which produced 2.74 g of biomass from 100 ml followed by coconut milk broth (2.14 g), oat meal broth (0.92 g) and potato dextrose broth (0.74 g). The least suitable one for biomass production was malt extract broth producing only 0.55 g.

Under static culture also jackfruit seed kernel broth produced maximum biomass of 6.19 g/100 ml which was about double the biomass obtained in shake culture. The coconut milk broth also produced higher biomass (2.91 g) when compared to the other broth. The potato dextrose

Table 13 Growth of *Calocybe* culture (OE-349) in different liquid broth

Sl No	Media	Biomass(g/100ml)*	
		Shake culture	Static culture
1	Potato dextrose broth	0.74	1.73
2	Malt extract broth	0.55	0.19
3	Oat meal broth	0.92	1.14
4	**Jack fruit seed kernel broth**	**2.74**	**6.19**
5	Coconut milk broth	2.14	2.91
	CD (0.05)	0.048	0.059

*Average of four replications

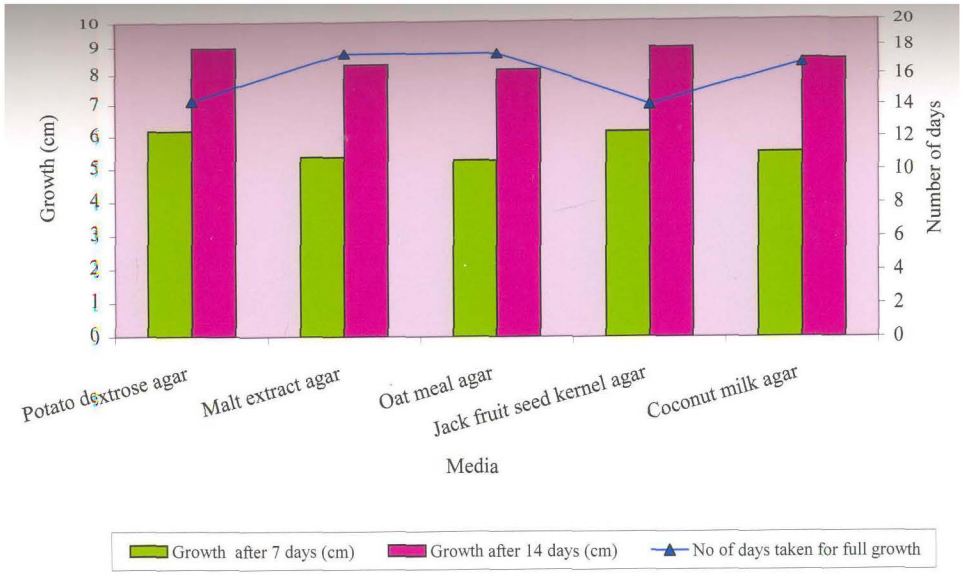


Fig. 11 Growth of *Calocybe* in different solid media

broth could support the production of 1.74 g biomass, double than that produced by shake culture. Oatmeal broth also produced higher biomass of 1.14 g under static culture. Similar to shake culture studies, in static culture also malt extract broth was found to be the least effective for growth of *Calocybe* (Fig. 12 and Plate 8a and 8b).

4.7.3 Growth of *Calocybe* in Different Carbon Sources

Seven different carbon sources namely, dextrose, fructose, galactose, inositol, mannitol, sucrose and lactose were tested for their efficacy in biomass production of *Calocybe*. The results showed that fructose was the best carbon source producing maximum biomass of 0.61 g followed by dextrose (0.29 g) and mannitol (0.24 g). The other carbon sources *viz.*, galactose, and inositol produced 0.22g, and 0.17 g respectively. Lactose and sucrose were found to be the least suitable, producing 0.12g, and 0.15g of biomass (Table 14, Fig. 13 and Plate 9).

4.7.4 Influence of Different Nitrogen Sources on the Growth of *Calocybe*

The nitrogen sources evaluated include both organic and inorganic types. Inorganic nitrogen sources tried were sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride and ammonium carbonate. Organic sources included were beef extract and peptone.

Organic nitrogen was found to be a better source of nitrogen than the inorganic sources. Peptone was the best nitrogen source producing the highest biomass of 0.49 g followed by beef extract with 0.27 g. Among the nitrate sources potassium nitrate was better compared to sodium nitrate and ammonium nitrate and they produced 0.24 g, 0.22 g and 0.12 g of biomass respectively. Among the various ammonium sources, ammonium chloride (0.14 g) was on par with ammonium nitrate and ammonium carbonate with respect to the biomass production. Of these seven nitrogen sources ammonium carbonate was the nitrogen source which contributed least to the biomass production (Table 15, Fig.14 and Plate 10).

Table 14 Growth of *Calocybe* culture in different carbon sources

Sl. No	Carbon sources	Biomass (g/50ml)*
1	Dextrose	0.29
2.	**Fructose**	**0.61**
3.	Galactose	0.22
4.	Inositol	0.17
5.	Mannitol	0.24
6.	Sucrose	0.15
7.	Lactose	0.12
	CD (0.05)	0.032

* Average of three replications

Table 15 Growth of *Calocybe* culture in different nitrogen sources

Sl No	Nitrogen sources	Biomass (g/50ml)*
1	Sodium nitrate	0.22
2.	Potassium nitrate	0.24
3.	Ammonium nitrate	0.12
4.	Ammonium chloride	0.14
5.	Ammonium carbonate	0.08
6.	Beef extract	0.27
7.	**Peptone**	**0.49**
	CD (0.05)	0.111

* Average of three replications

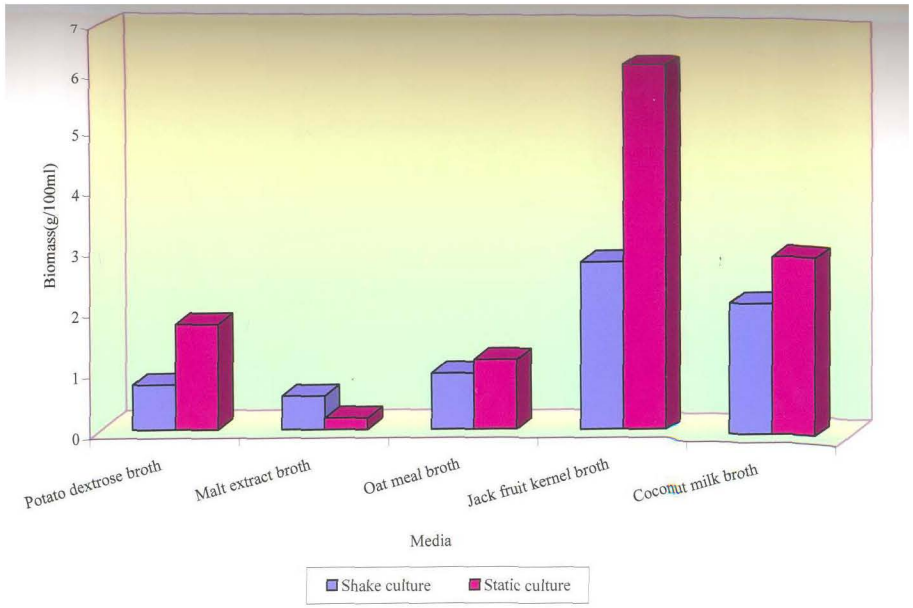


Fig. 12 Growth of *Calocybe* in different liquid broth

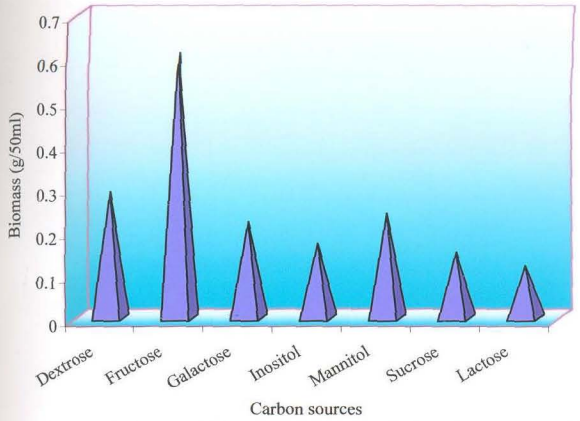


Fig. 13 Influence of carbon sources on the mycelial growth of *Calocybe*

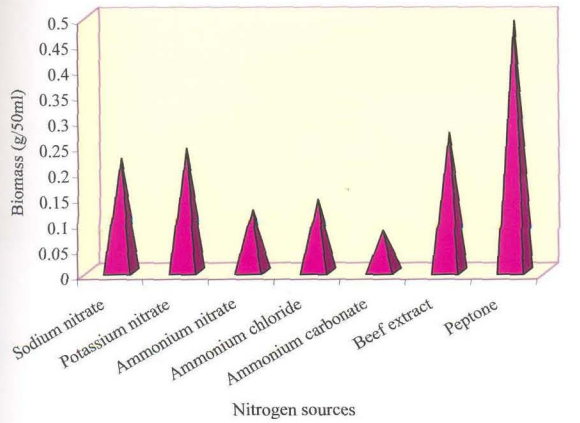
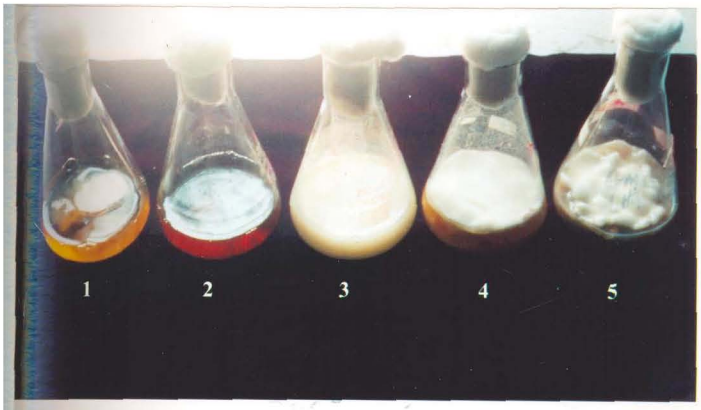


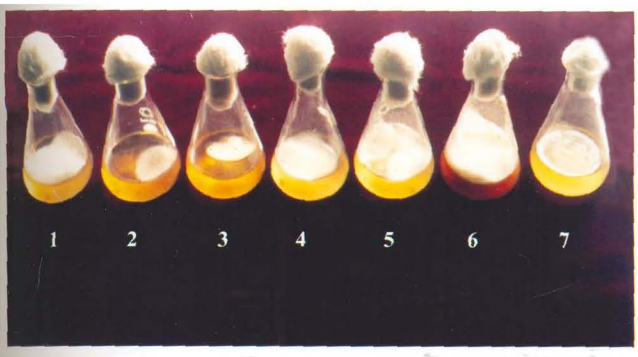
Fig. 14 Influence of nitrogen sources on the mycelial growth of *Calocybe*



1. Potato dextrose broth 2. Malt extract broth 3. Oat meal broth
4. Jackfruit seed kernel broth 5. Coconut milk broth

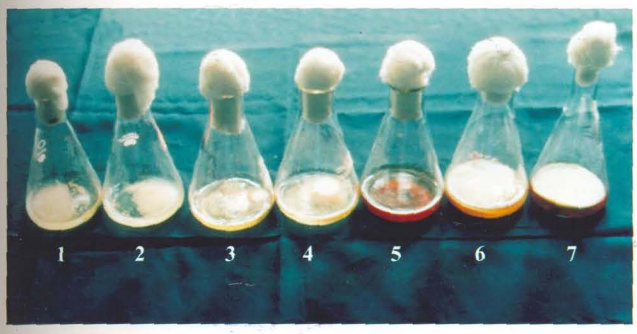
Plate 8a Growth of *Calocybe* in liquid broth

Plate 8b Jackfruit seed kernel broth



1. Mannitol 2. Inositol 3. Sucrose 4. Galactose
5. Dextrose 6. Fructose 7. Lactose

Plate 9 Growth of *Calocybe* in different carbon sources



1. Potassium nitrate 2. Sodium nitrate 3. Ammonium nitrate
4. Ammonium chloride 5. Ammonium carbonate 6. Peptone 7. Beef extract

Plate 10 Influence of different nitrogen sources on the growth of *Calocybe*

4.7.5 Effect of Different H^+ ion Concentration on the Growth of *Calocybe*

Nine different H^+ ion concentration (pH) ranging from 4 – 8.5 were tested for their efficacy in the production of biomass as given in Table 16.

It was observed that maximum biomass was obtained at pH 5.5 as in Table 16. There was a steady increase in biomass production from pH 4.0 to 5.5, followed by reduction in biomass. Least biomass was obtained at pH 8.5 (0.14 g) (Fig. 15 and Plate11). There was significant difference between pH 5.5 and 6.0 but not with 6.5. The biomass production of various pH 6.0, 6.5, 7.0, 7.5, 8 and 8.5 were 0.53, 0.47, 0.35, 0.295, 0.21 and 0.14 g respectively.

There was significant difference between pH 5.5 and 6.0. The pH 6.5, 7.0, 4.0, 7.5 were on par whereas pH 8.5 and 8.0 were also equal in their effect.

At pH 5.5 biomass production was four times that of 8.5, pH 6 and 6.5 had a biomass of three times that of pH 8.5.

It was observed that *Calocybe* prefers a pH of 5.5 – 6.0 rather than the alkaline range where the biomass sharply declined. Even highly acidic environment *i.e.*, pH 4.0 was not favourable for the production of biomass.

4.8 IMPROVEMENT OF PRODUCTION TECHNOLOGY

4.8.1 Screening and Selection of Substrates based on Different Sterilization Procedures

Three different locally available substrates, namely paddy straw, spent mushroom substrate (SMS), and coir pith were subjected to three different sterilization procedures *viz.*, boiling, solarisation and chemical treatments.

Table 16 Growth of *Calocybe* culture in different pH

Sl. No	pH	Biomass (g/50ml)*
1	4.0	0.33
2.	5.0	0.39
3.	**5.5**	**0.64**
4.	6.0	0.53
5.	6.5	0.46
6.	7.0	0.35
7.	7.5	0.29
8.	8.0	0.21
9.	8.5	0.14
	CD (0.05)	0.106

* Average of three replications

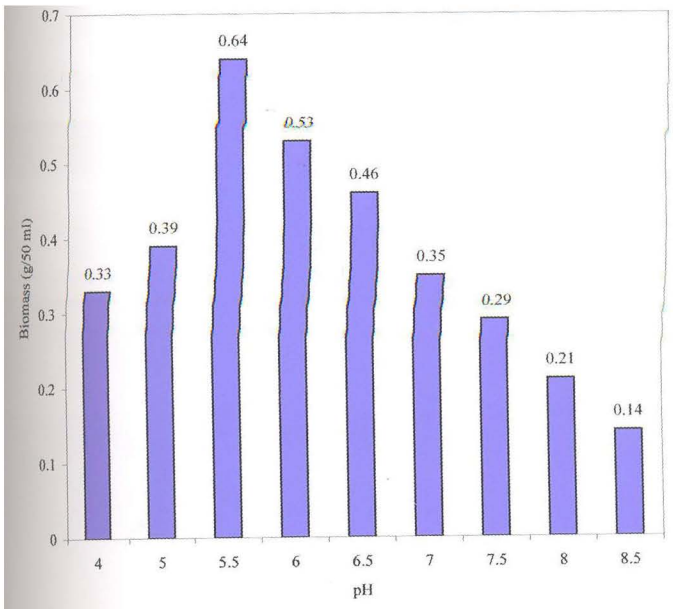


Fig. 15 Influence of pH on mycelial growth of *Calocybe*

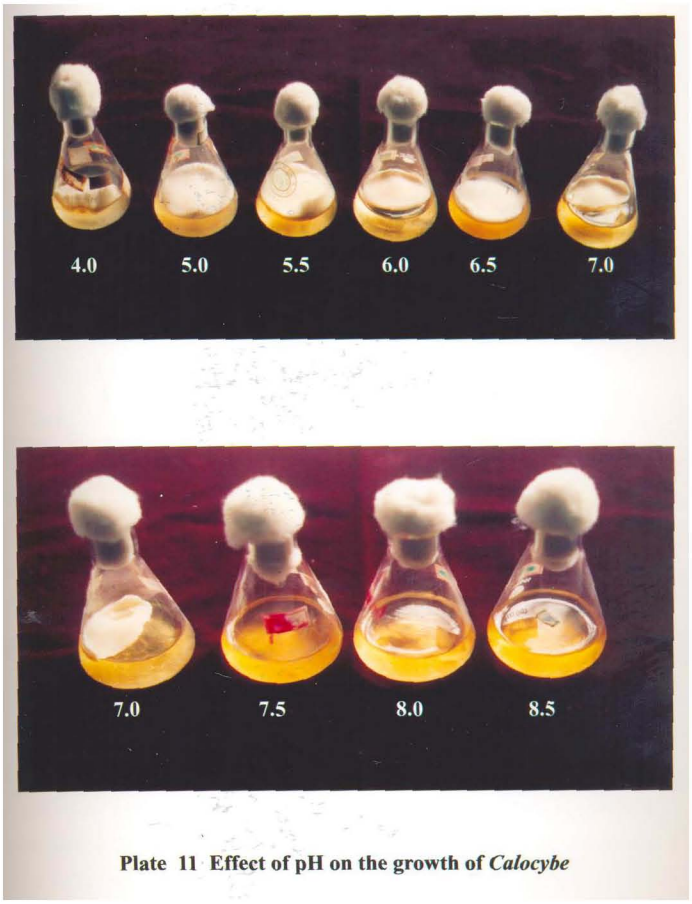


Plate 11 Effect of pH on the growth of *Calocybe*

4.8.1.1 Mycelial Characters and Time for the Production of Mushrooms

4.8.1.1.1 Nature of Mycelial Growth

There was no significant difference in the nature of mycelial growth of mushroom with the different substrates used. But when the sterilization method is considered, solarisation gave the best growth both in paddy straw and spent mushroom substrate (Table 17).

4.8.1.1.2 Time for Spawn Run

It was found that there was no significant difference between the substrates with respect to time for spawn run. Among the different sterilization procedures solarisation was the best which took minimum period of 18.2 days for spawn run when compared to boiling (20.40) and chemical (21.20) respectively (Fig. 16).

4.8.1.1.3 Days Taken for Initial Production

There was no significant difference between the substrates namely paddy straw and spent mushroom substrate with respect to days taken for primordial initiation. The coirpith substrate subjected to solarisation and boiling did not produce any sporophore due to contamination by *Trichoderma.* Among the various sterilization techniques solarisation resulted in early production of *Calocybe* initials (8.2 days) when compared to boiling (11.2 days) and chemical method (10.3 days).

The interaction studies conducted showed that paddy straw sterilized by solarisation and solar sterilized SMS took minimum period of 8.20 days for spawn run followed by chemically sterilized paddy straw and SMS with 10.40 and 10.20 days respectively. The sterilization techniques *i.e.*, boiling took comparatively more time for spawn run (11.8 days). The results are presented in Table 17 and Fig.16.

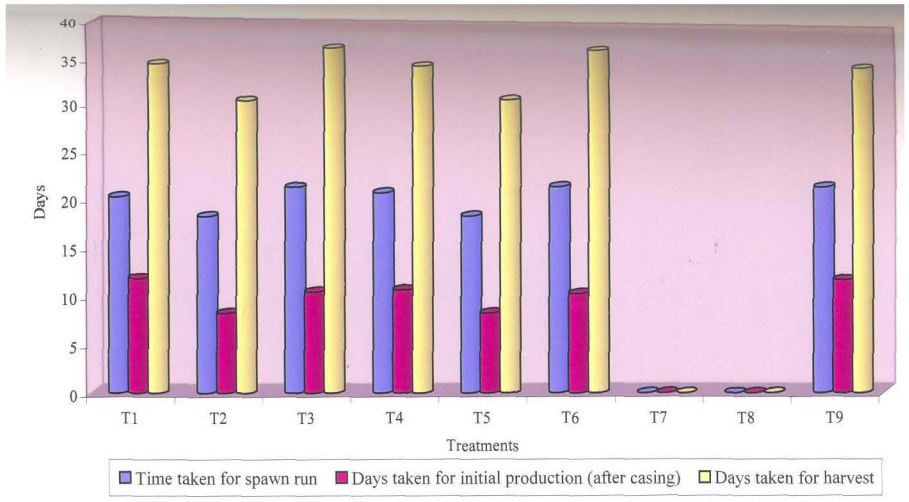
Table 17 **Effect of substrate sterilization on the mycelial growth and time taken for mushroom production**

Sl No	Treatments	Nature of mycelial growth	Time taken for spawn run*	Days taken for initial production (after casing)*	Days taken for harvest*
1.	T_1- Paddy straw +boiling	+++	20.20	11.80	34.60
2.	T_2- Paddy straw + solarisation	+++++	18.20	8.20	30.40
3.	T_3- Paddy straw + chemicals	++++	21.20	10.40	36.80
4.	T_4- Spent mushroom substrate + boiling	+++	20.60	10.60	34.60
5.	T_5- Spent mushroom substrate + solarisation	+++++	18.20	8.20	30.60
6.	T_6- Spent mushroom substrate + chemical	+++	21.20	10.20	36.80
7.	T_7- Coir pith +boiling	-	-	-	-
8.	T_8- Coir pith + solarisation	-	-	-	-
9.	T_9- Coirpith + chemicals	+++	21.20	11.60	34.80
	CD(0.05) for substrate		NS	NS	NS
	CD(0.05) for sterilization procedure		0.92	1.08	1.56
	CD for interaction		NS	NS	NS

*Average of five replications

Mean values

	Time taken for spawn run	Days taken for initial production	Days taken for harvest
Paddy straw	19.87	10.13	33.93
SMS	20.00	9.67	34.00
Coir pith	21.20	11.60	34.80
Boiling	20.40	11.20	34.60
Solarisation	18.20	8.20	30.50
Chemicals	21.20	10.30	34.80



T_1- Paddy straw +boiling
T_2- Paddy straw + solarisation
T_3- Paddy straw + chemicals
T_4- Spent mushroom substrate + boiling
T_5- Spent mushroom substrate + solarisation
T_6- Spent mushroom substrate + chemical
T_7- Coir pith +boiling
T_8- Coir pith + solarisation
T_9- Coirpith + chemicals

Fig. 16 Influence of sterilization methods of the substrates on mushroom production

4.8.1.1.4 Days for Harvest

The days taken for harvest did not vary significantly with the substrates paddy straw and SMS. Solarisation of substrates reduced the time taken for harvest *i.e.*, 30.50 days followed by boiling (34.60 days) and chemical method (36.80 days).

The effect of different substrate and sterilization procedure on time taken for harvest showed that the minimum period requirement was in case of solarised paddy straw (30.40), and spent mushroom substrate (30.60 days). For mushroom harvest paddy straw and spent mushroom substrate sterilized by boiling took 34.60 days. The chemically sterilized paddy straw and spent mushroom substrate took longer period of 36.80 days for harvest. Chemically sterilized coirpith took 34.80 days for harvest (Table 17 and Fig. 16).

4.8.1.2 Yield Performance of Different Substrates and Sterilization Procedures

4.8.1.2.1 Sporophores Harvested and Yield

Substrates *viz.*, paddy straw, SMS and coirpith showed significant variation with respect to the number of sporophores harvested. Spent mushroom substrate produced 8.13 mushrooms where as paddy straw gave 5.40 and coirpith 3.80 numbers. Among the sterilization techniques, solarisation yielded maximum number of sporophores (11.40) followed by boiling and chemical treatment which were on par.

The interaction studies revealed the result of higher number of sporophores (14.20) in the case of solarised spent mushroom substrate(T_5), followed by solarised paddy straw with 8.60 sporophores. The coirpith sterilized by chemicals and boiling (T_7, T_8) did not produce any sporophores. The treatment T_6, T_4, T_1, T_9 and T_3 were on par giving 5.60, 4.60, 4.40, 3.80 and 3.20 sporophores respectively (Table 18).

Table 18 Effect of substrate and sterilization procedures on the yield and other characters

Sl No	Treatment	No of sporophores	Yield (g/kg substrate)	Biological efficiency (%)	Mortality (%)	Average fruit body weight (g)	Contaminants observed
1.	T_1- Paddy straw + boiling	4.40	145	14.5	77.93 (61.95)	18	*Coprinus*
2.	T_2- Paddy straw + solarisation	8.60	**450**	45.0	**59.30 (50.40)**	36	-
3.	T_3- Paddy straw + chemicals	3.20	203	20.3	67.49 (55.21)	17	*Coprinus*
4.	T_4- Spent mushroom substrate + boiling	4.60	216	21.6	70.80 (57.27)	26	*Coprinus, Trichoderma*
5.	T_5- Spent mushroom substrate + solarisation	14.20	**602**	60.2	**56.73 (48.85)**	42	-
6.	T_6- Spent mushroom substrate + chemical	5.60	223	22.3	77.79 (61.86)	21	-
7.	T_7- Coir pith +boiling	-	-	-	0	-	*Trichoderma*
8.	T_8- Coir pith + solarisation	-	-	-	0	-	*Trichoderma*
9.	T_9- Coirpith + chemicals	3.8	223	22.3	81.60(65.03)	29	*Coprinus, Trichoderma*
	CD(0.05) for substrate	1.21	33.02	3.30	5.14		
	CD(0.05) for sterilization procedure	1.48	40.44	4.04	6.30	14.86	
	CD for interaction	2.09	57.19	5.72	8.91		

*Average of five replications

Mean value

	No of sporophores	Yield	Biological efficiency	Mortality	Average fruit body weight
Paddy straw	5.40	266.00	26.60	68.53 (55.83)	23.67
SMS	8.13	347.00	34.70	68.75 (55.99)	29.67
Coir pith	3.80	223.00	22.30	65.06 (53.21)	29.00
Boiling	4.50	180.50	18.05	74.45 (59.61)	22.00
Solarisation	11.4	526.00	52.60	58.06 (49.62)	39.00
Chemicals	4.2	213.00	21.30	72.79 (58.54)	22.30

Figures in parentheses are angular transformed values

4.8.1.2.2 Yield and Biological Efficiency

There was a significant difference in yield with respect to the substrates used. Spent mushroom substrate gave higher yield (347 g) followed by paddy straw (266 g) and coirpith (223 g). The solarisation of substrate was the most effective in producing maximum yield. Solarised SMS produced maximum yield of 602 g which was significantly different from solarised paddy straw which gave 450g (Plate 12). Paddy straw subjected to boiling produced the lowest yield of 145g. Chemically sterilized paddy straw, SMS, coirpith and SMS subjected to boiling were on par (Fig. 17).

Solarised spent mushroom substrate gave the highest BE of 60.2 per cent followed by solarised paddy straw with 45 per cent biological efficiency. Lowest BE was noticed in the treatment T_7 and T_8. Biological efficiencies of others varied from 14.5-22.3 per cent (Table 18).

4.8.1.2.3 Mortality Percentage

The coirpith used as substrate resulted in no mortality. Paddy straw and spent mushroom substrate resulted in mortality, the percentage being on par. Solarisation of substrates resulted in minimum mortality percentage (58.06 %) and boiling and chemical sterilization were on par with 74.45 and 72.79 per cent respectively.

The mortality percentage was minimum in T_5 with 56.73 per cent and maximum in T_7 and T_8 (100 %) (Table 18 and Fig. 18).

4.8.1.2.4 Average Fruit Body Weight

The substrate variation did not significantly affect the fruit body weight. Solarised substrate gave the maximum fruit body weight followed by chemical sterilization and boiling.

The interaction studies indicated that T_5 and T_2, (solarised spent mushroom substrate and paddy straw) gave the maximum fruit body weight

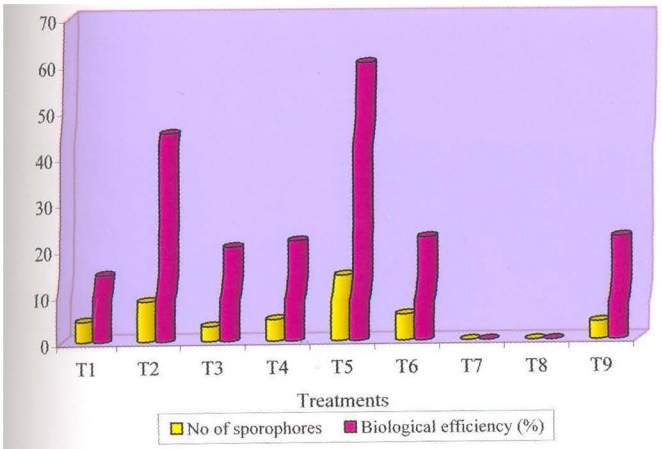


Fig. 17 Effect of substrate and sterilization procedures on the yield

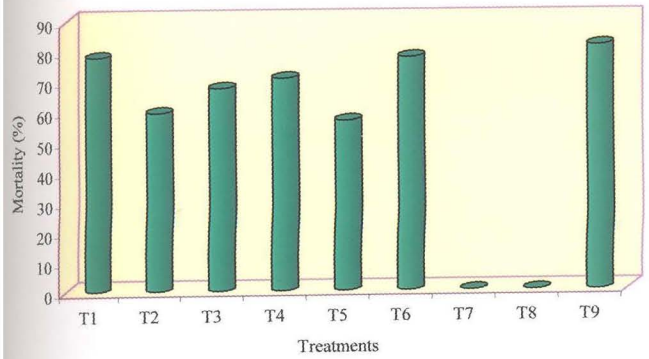


Fig. 18 Combination effect of substrate and sterilization procedures on mortality of primordia

T_1- Paddy straw +boiling
T_2- Paddy straw + solarisation
T_3- Paddy straw + chemicals
T_4- Spent mushroom substrate + boiling
T_5- Spent mushroom substrate + solarisation
T_6- Spent mushroom substrate + chemical
T_7- Coir pith +boiling
T_8- Coir pith + solarisation
T_9- Coirpith + chemicals

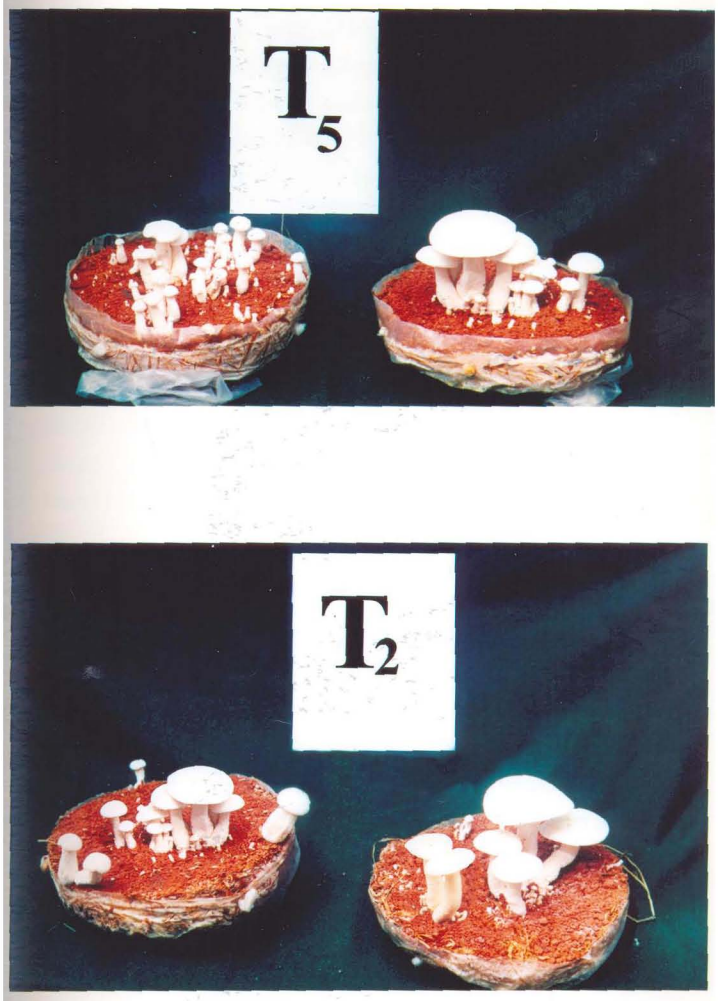


Plate 12 Comparative efficiency of different solarised substrates on yield

of 42g and 36 g respectively. The minimum weight was obtained in T_3 with 17 g which was on par with T_1 (Table 18).

4.8.1.2.5 Contaminants

It was observed that coirpith subjected to boiling and solarisation was contaminated with *Trichoderma*. Chemically sterilized coir pith had *Coprinus* along with *Trichoderma* as contaminants. The solarised beds of SMS and paddy straw were free of any contaminants. In boiled substrates weed mould *Coprinus* was noticed (Table 18).

4.8.2 Effect of Supplements on the Yield of *Calocybe*

The substrate and the sterilization technique selected from 4.7.1 was used for further studies. It was observed that spent mushroom substrate sterilized by solarisation gave maximum yield of *Calocybe*. The solarised spent mushroom substrate of one kg dry weight was treated with various supplements *viz.*, neem cake, rice bran, vermicompost and ferrous sulphate. The supplements were mixed @ 20 g/kg substrate and 40 g/kg substrate except ferrous sulphate which was applied at 5 ppm and 10 ppm concentrations (Table 19).

4.8.2.1 Number of Sporophores Harvested

Four different supplements were tested for their effect on yield. It was observed that rice bran was superior to all other supplements. The supplementation of spent mushroom substrate (SMS + paddy straw in 1 : 1) with rice bran gave maximum number of sporophores (13.50) compared to the control (5.50). Among the various treatments lesser number of sporophore were obtained from $FeSO_4$ (5 ppm) and vermicompost two per cent. Supplementation of substrate with neem cake and vermicompost gave lowest number of sporophores *i.e.*, 4.00 nos/bed.

It was also noted that treatments $FeSO_4$ (5 ppm), neem cake 2 per cent and neem cake 4 per cent were on par giving 3.25, 4.00, and 4.00 number of sporophores respectively. Treating the substrates with vermicompost 4 per cent,

Table 19 Effect of supplements on the yield of *Calocybe*

Sl No	Treatments	Sporophores harvested*		Yield(g/kg substrate)*		Percentage increase/decrease over control	Biological efficiency (%)*		Contaminants observed
		Number	Mean value	Yield	Mean value		BE	Mean value	
1.	T_1- Neem cake 2%	4.00	4.00	88.75	118.13	-72.527	8.87	11.81	*Trichoderma*
2.	T_2 - Neem cake 4%	4.00		147.50		-53.575	14.75		*Trichoderma*
3.	T_3 - Rice bran 2%	11.75	13.50	403.75	523.13	27.395	40.3	52.31	-
4.	T_4 - Rice bran 4%	**15.25**		**642.50**		102.438	**64.25**		-
5.	T_5 -Ferrous sulphate 5ppm	3.25	4.50	78.75	113.75	-74.785	7.88	11.38	*Trichoderma*
6.	T_6 -Ferrous sulphate 10ppm	5.75		148.75		-52.363	14.88		-
7.	T_7 – Vermicompost 2%	2.75	4.00	113.75	152.50	-63.995	11.38	15.25	*Trichoderma*
8.	T_8 – Vermicompost 4%	5.25		191.25		-40.2	19.12		*Trichoderma*
9.	T_9 – Control	5.50		320			32.00		
	CD(0.05) for supplement	1.176		38.699			3.869		
	CD(0.05) for rates	1.664		54.729			5.473		
						23.771			

*Average of four replications

control and $FeSO_4$ (10 ppm) were similar in their efficacy in producing 5.25, 5.50, and 5.75 number of sporophores respectively. Rice bran @ 2 per cent and rice bran @ 4 per cent were significantly different from the other treatments. Rice bran was found to be a superior supplement when compared to vermicompost, neem cake and ferrous sulphate.

Among the rates of supplements rice bran @ 4 per cent was superior than rice bran applied @ 2 per cent. Rice bran when applied @ 4 per cent gave 15.25 sporophores whereas 2 per cent yielded 11.75. There was no significant difference between neem cake at 2 per cent and 4 per cent. The application of ferrous sulphate @ 5ppm and 10 ppm showed significant difference, with better effect on 10 ppm *i.e.*, 5.75 number of sporophores and 3.25 numbers in 5 ppm. Similarly supplementation of substrate with vermicompost 4 per cent yielded five sporophores whereas 2 per cent gave three sporophores. In general it was observed that higher concentration of supplements enhanced sporophore production in *Calocybe*.

4.8.2.2 Yield

The yield obtained from rice bran (523.13 g) was significantly higher than that of other supplements *viz.*, neem cake, vermicompost, ferrous sulphate and control. Vermicompost supplementation gave a yield of 152.50 g, followed by neem cake supplementation with 118.13 g. Even though ferrous sulphate provided maximum number of sporophores than neem cake, it was not efficient in producing higher yield. The unsupplemented substrate gave higher yield than neem cake, vermicompost and ferrous sulphate.

A similar trend was observed in the number of sporophores harvested. Rice bran supplementation at 4 per cent rate out yielded 2 per cent application. The rice bran applied @ 4 per cent gave 642.50 g whereas at 2 per cent yield was 403.75 g. The application of vermicompost @ 4 per cent gave 191.25 g. Supplementation of all other substrates at a higher concentration gave enhanced yield. Among the different supplementation it

was concluded that rice bran 4 per cent as superior and ferrous sulphate 5 ppm as inferior supplements (Table 19, Fig. 19 and Plate 13).

Percentage increase over control among various treatments indicated that rice bran application resulted in increased yield where was other treatments resulted in reduction of yield. All the other supplements showed reduced yield when compared to the control hence percentage over control was negative. Rice bran (4 %) gave 102.43 per cent increase whereas rice bran two per cent gave 27.40 per cent. Maximum reduction in yield of -74.78 per cent was noticed in treatment T_5 (Fig. 20).

4.8.2.3 Biological Efficiency (Per cent)

Biological efficiency of the various treatments varied widely. It was observed that the BE varied from 8.87 – 64.25 per cent. The maximum biological efficiency was noticed in substrate (SMS) supplemented with rice bran @ four per cent (64.25) followed by rice bran @ two per cent (40.37). The substrate without any supplementation served as control has given better BE (32.00 %) than the other supplements. Vermicompost four per cent showed a BE of 19.12 per cent. The substrate supplementation with $FeSO_4$ 10 ppm, neem cake four per cent, vermicompost two per cent, neem cake two per cent which were on par had 14.88 per cent, 14.75, 11.38 and 8.88 per cent respectively. Lowest biological efficiency was noticed in supplementation of substrate with ferrous sulphate 5 ppm (Table 19).

4.8.2.4 Incidence of Contaminants

Supplementation of substrate increased the incidence of contaminants. Severe contamination of *Trichoderma* was noticed on SMS supplemented with neem cake and vermicompost. The contaminants were absent in the case rice bran supplementation. Higher rate of supplements favoured heavy incidence of *Trichoderma* except rice bran (Table 19 and Plate 14).

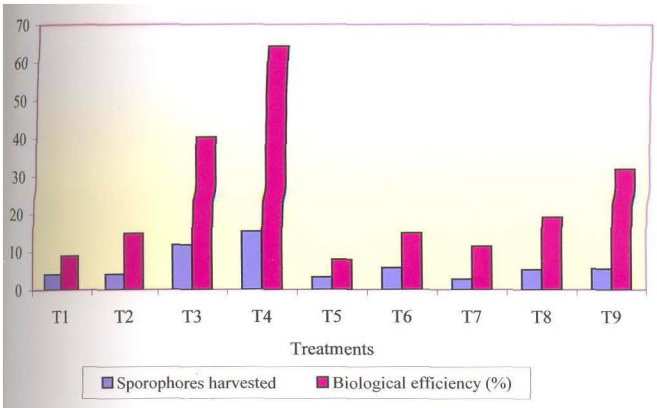


Fig. 19 Influence of supplements on the yield of *Calocybe*

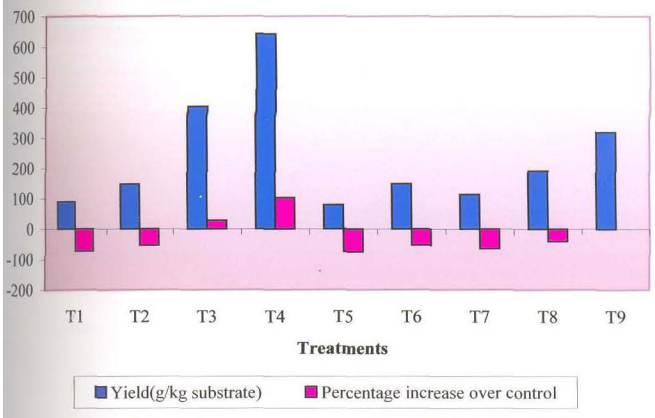


Fig. 20 Effect of supplements on the yield and percentage increase over control of *Calocybe*

T_1 – Neem cake 2%
T_2 – Neem cake 4%
T_3 – Rice bran 2%
T_4 – Rice bran 4%
T_5 – Ferrous sulphate 5ppm
T_6 – Ferrous sulphate 10ppm
T_7 – Vermicompost 2%
T_8 – Vermicompost 4%
T_9 – Control

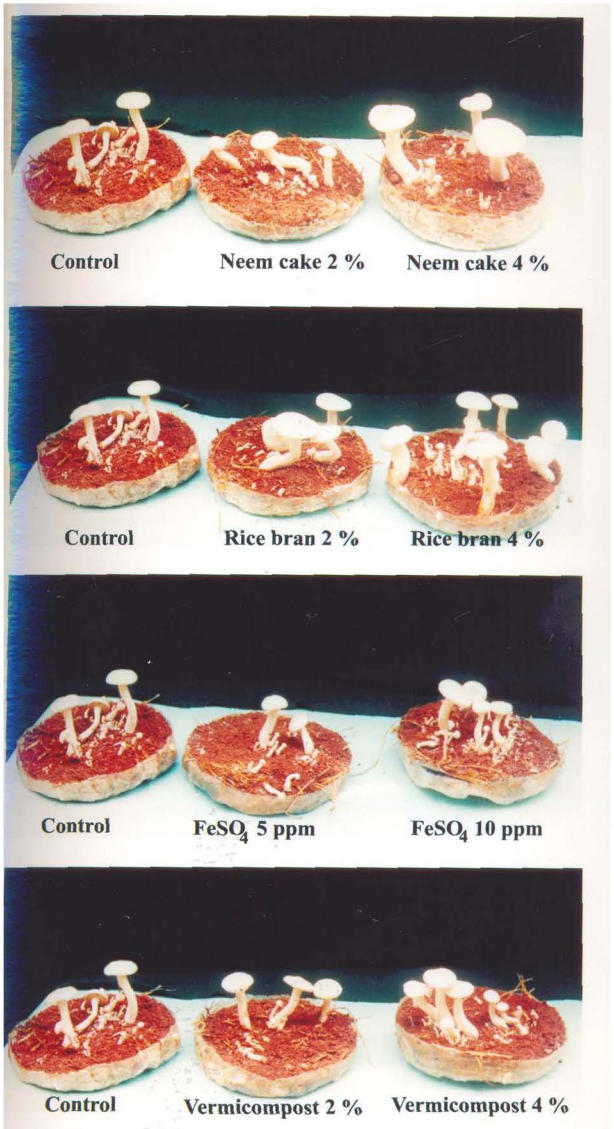


Plate 13 Effect of supplements on the yield of *Calocybe*

Plate 14 Contamination in supplemented substrate

4.8.3 Effect of Casing on Yield Parameters of *Calocybe*

The study was conducted to evaluate the various casing materials *viz.*, coir pith and soil, vermicompost and soil, clay and soil, control (sand, soil and calcium carbonate (Table 20). The beds were prepared with solarised spent mushroom substrate supplemented with rice bran (4.00 per cent).

4.8.3.1 Number of Sporophores Harvested

The beds were cased with sand, soil and calcium carbonate mixture (control) after 21 days after spawning (15.65), out yielded other casing materials, with vermicompost soil mixture and coirpith soil mixture which were giving 7.91 and 3.25 sporophores respectively. The least effective casing material was clay and soil as it did not yield any sporophores. (Table 20).

4.8.3.2 Yield

The yield was higher in control than the other treatments. The bed cased with sand, soil and calcium carbonate mixture gave 663.12 g of mushroom, followed by beds cased with vermicompost soil mixture (272.99 g) and those with coirpith soil mixture as casing material with 62.52 g (Table 20, Fig 21 and Plate 15).

4.8.3.3 Biological Efficiency (Per cent)

The biological efficiency is highest in control treatment with 66.31 per cent followed by vermicompost soil mixture cased beds (27.29 %).In clay soil mixture cased beds there was no sporophore yield hence had 0% biological efficiency.

Since the yield from control beds were higher percentage increase over control in the case of other treatments were negative.

4.8.3.4 Contaminants

It was also observed in the study that increased incidence of competitor mould *Trichoderma* in beds cased with coirpith - soil mixture and vermicompost - soil mixture (Table 20 and Plate 16).

Table 20 Effect of casing on the yield of *Calocybe*

Sl. No	Treatments	No of sporophores harvested	Yield (g/kg substrate)	Percentage increase over control	Biological efficiency (%)	Contaminants observed
1.	T_1- Coir pith + soil	3.25 (2.05)	62.52 (7.97)	-89.477	6.25 (2.17)	*Trichoderma*
2.	T_2 – Vermicompost + soil	7.91 (2.98)	272.99 (16.55)	-58.623	27.29 (10.69)	*Trichoderma*
3.	T_3 – Clay + soil	0 (1)	0 (1)	0	0(1)	-
4.	T_4 – Control (Sand + soil+ $CaCO_3$)	15.65 (4.08)	663.12 (25.77)	-	66.31 (8.20)	-
	CD(0.05)	0.429	2.243		4.630	

Figures in parentheses are $\sqrt{x+1}$ transformed values

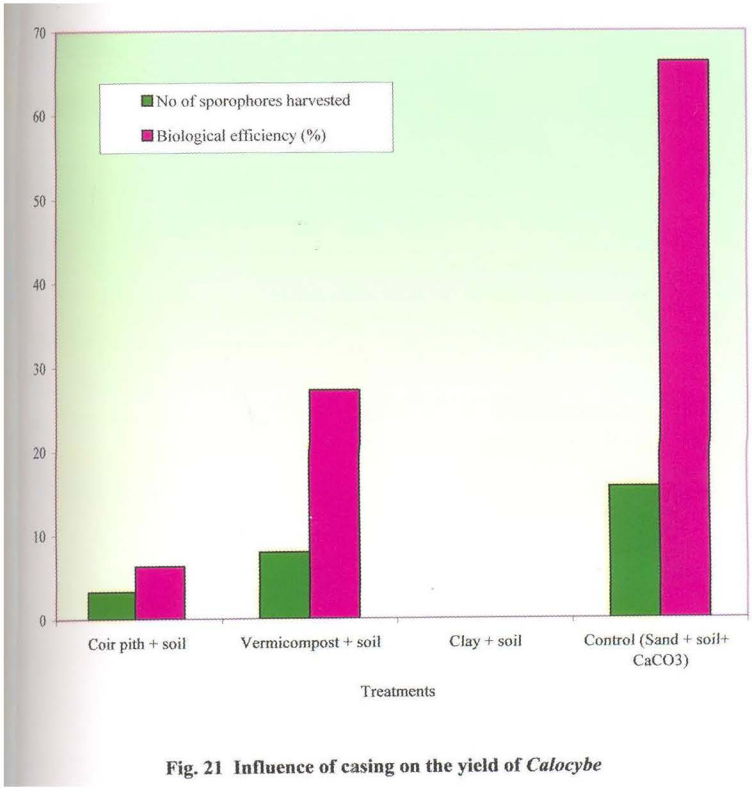


Fig. 21 Influence of casing on the yield of *Calocybe*

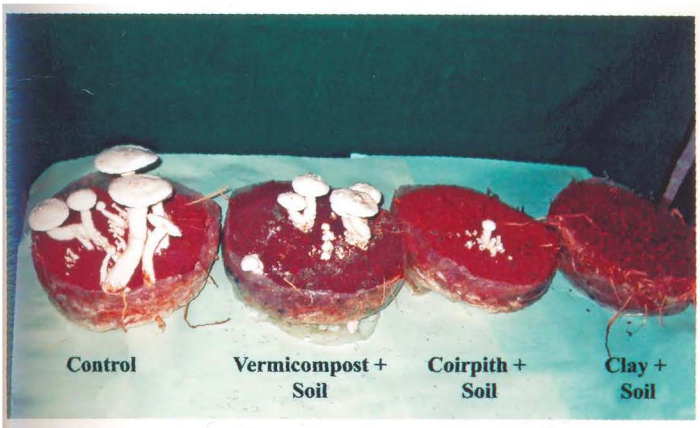


Plate 15 Effect of casing on yield

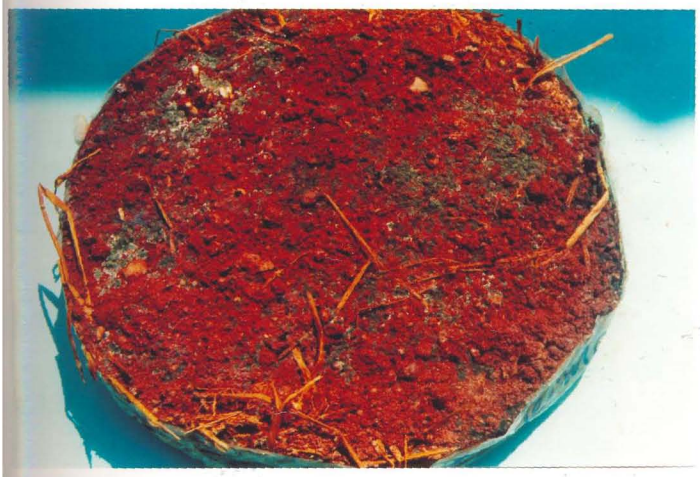


Plate 16 Contamination on cased beds

4.9 STRAIN IMPROVEMENT

4.9.1 Irradiation

4.9.1.1 Irradiation with UV Rays

The isolate of *Calocybe* (OE-349) was selected for irradiation studies. UV irradiation was done at two distances 5 cm and 10 cm from the source with 10, 15, 20 and 25 minutes of exposure. The irradiated cultures were maintained on PDA slants by periodical subculturing.

4.9.1.1.1 Radial Mycelial Growth of Irradiated Cultures of *Calocybe*

The cultures irradiated at different levels were tested for the proliferation of mycelia in potato dextrose agar medium and radial growth on the petridishes were observed. The study revealed that there was no significant difference between the distances from the source of UV. The time of exposure varied from 10-25 minutes indicating growth of isolates at 10, 20 minutes with 5.25 and 5.75cm of growth. The exposure for 15 minutes had better growth when compared to that of 25 minutes. The interaction of two factors indicated that irradiation at 5 cm for 20 min (T_3) resulted in lesser growth when compared to control. The treatments control, T_5, T_7 and T_1 were on par with 5.3cm, 5.53cm, 5.57cm, and 5.87 cm of growth respectively. The growth of irradiated cultures with T_4, T_6, T2 and T_8 were on par having better growth (Table 21). *Calocybe* cultures irradiated at 5cm and 10 cm for different periods have shown full growth in petridish after 14 days same as that of control (Fig. 22, Plates 17 and 18).

Nature of mycelial growth indicated that non-irradiated cultures had maximum fluffy growth when compared to irradiated culture. The culture irradiated at 10 cm for 15 minutes had growth similar to that was irradiated control. The cultures irradiated at T_1, T_3, T_5, T_7, and T_8 had medium growth. Feable growth was observed in time T_2 and T_4.

Table 21 Effect of UV irradiation on the growth of *Calocybe*

Treatment		Growth of culture in petridish 7 days (cm)*	Nature of mycelial growth
Distance	Period of exposure		
5 cm	10 min (T_1)	5.87	++++
	15 min (T_2)	6.27	+++
	20 min (T_3)	4.93	++++
	25 min (T_4)	6.03	+++
10 cm	10 min (T_5)	5.53	++++
	15 min (T_6)	6.13	+++++
	20 min (T_7)	5.57	++++
	25 min (T_8)	6.23	++++
Control		5.33	+++++
CD (0.05) for distance		NS	
CD (0.05) for period of exposure		0.33	
CD (0.05) for interaction		0.47	

*Average of three replications

		Mean value
Distance (cm)	5.00	5.78
	10.00	5.87
Period (min)	10	5.70
	15	6.20
	20	5.25
	25	6.13

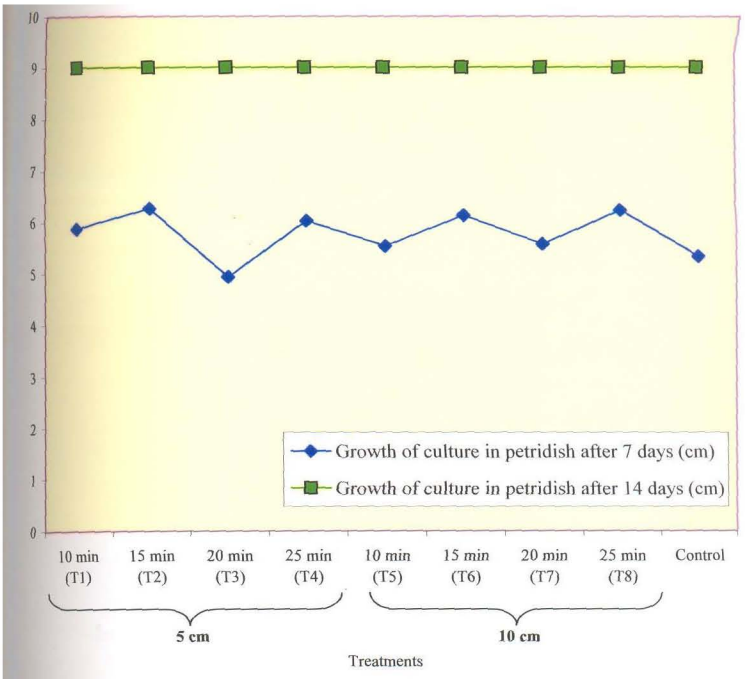
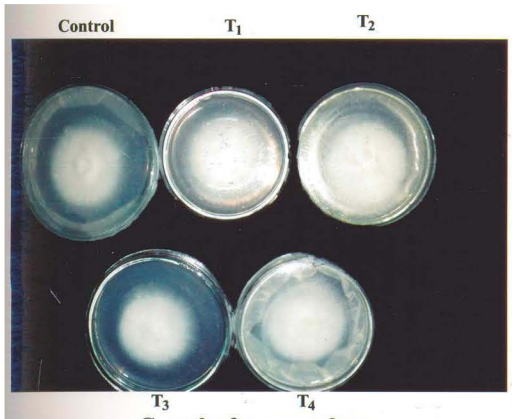
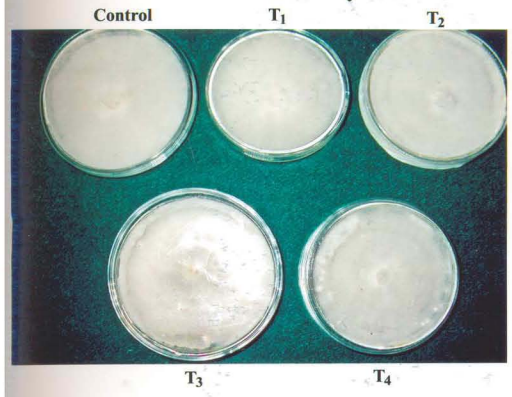


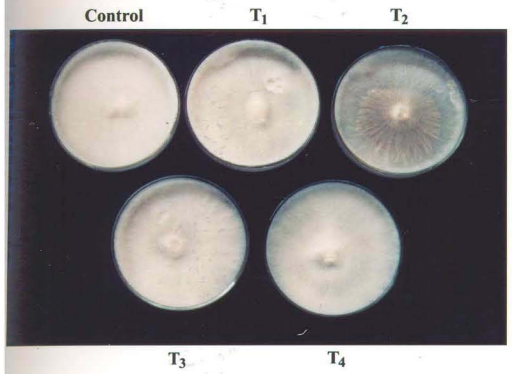
Fig. 22 Influence of UV irradiation on the growth of *Calocybe*



Growth after seven days

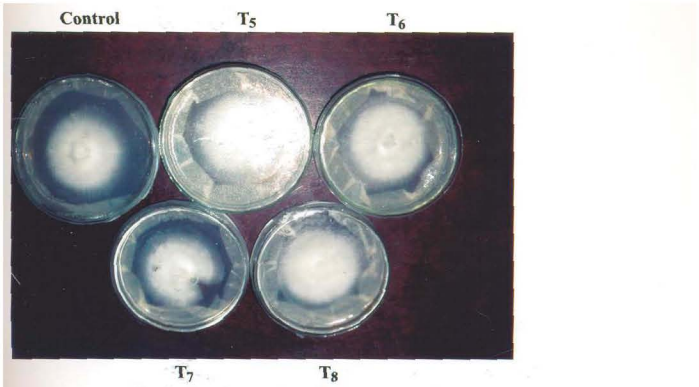


Growth after 14 days

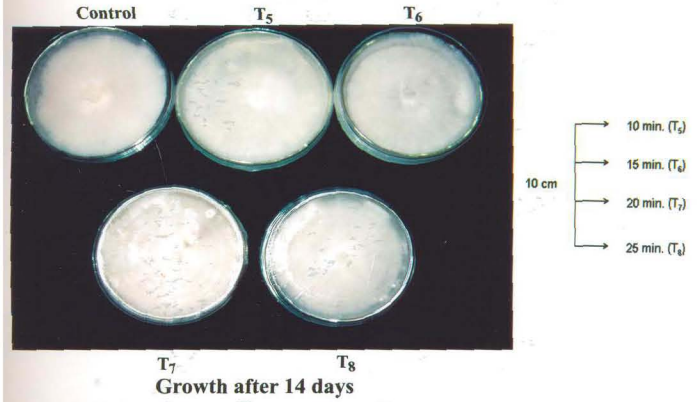


Nature of growth

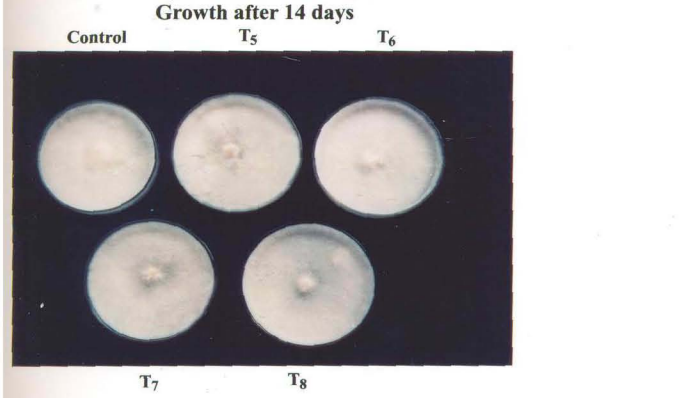
Plate 17 Influence of UV irradiation at 5 cm from the source on the growth of *Calocybe*



Growth after seven days



Growth after 14 days



Nature of growth

Plate 18 Influence of UV irradiation at 10 cm from the source on the growth of *Calocybe*

4.9.1.1.2 Evaluation of Yield Performance of Irradiated Culture

4.9.1.1.2.1 Time for Mushroom Production

The time taken for mushroom production with irradiated cultures were higher than that of non-irradiated cultures. But there was not significant difference between distance from the source and time of exposure of culture. Since the time taken is higher than the control it is not preferable (Table 22).

4.9.1.1.2.2 Number of Sporophores Harvested

There was a significant difference between the distances from the source with respect to the number of sporophores obtained at 3.25 numbers at 5 cm, 6.92 at 10 cm. On the basis of time also there was significant difference with T_3 (20 minutes).

The interaction between distance and time of exposure revealed that cultures irradiated at 10 cm away from source for 15, 20 min yielded maximum number of sporophores of 7.67 and 9.67. The treatments T_9, T_5 and T_8 were on par indicating effect of irradiation at 10 cm from sources. The cultures irradiated at 5cm away from source for different periods have shown similar effect (Table 22).

4.9.1.1.2.3 Yield

The yield performances of the irradiated cultures were compared with the control, using a non irradiated parent culture (Table 25). There was a significant difference between the distance from source of UV light and time of exposure. Exposure of cultures to 10 cm distance from source showed enhanced yield when compared to 5 cm distance. The yield increase due to different time of exposure indicated that exposure for 20 minutes and 15 minutes had significant effect when compared to 10 minutes and 25 minutes.

The interaction study indicated that non-irradiated culture had maximum yield when compared with irradiated cultures. The treatments T_4

Table 22 Efficacy of UV irradiation on the yield of *Calocybe*

Treatment		Time taken for mushroom production (days)*	No of sporophores harvested*	Yield(g/kg substrate)*	Percentage increase/ decrease over control	Biological efficiency (%)*
5 cm	10 min (T_1)	36.00	3.33	158.30	-69.26	15.83
	15 min(T_2)	35.00	2.67	108.33	-78.80	10.83
	20 min(T_3)	36.00	3.67	151.67	-71.37	15.17
	25 min(T_4)	36.00	3.33	65.00	-87.86	6.50
10 cm	10 min (T_5)	34.00	5.67	201.67	-61.84	20.17
	15 min (T_6)	35.00	7.67	433.33	-16.54	43.33
	20 min (T_7)	35.50	9.67	496.67	-4.34	49.67
	25 min (T_8)	36.00	4.67	283.33	-45.79	28.33
Control		33.50	6.67	521.00		52.10
CD(0.05) for distance		NS	1.03	48.28		4.83
CD(0.05) for period		NS	1.46	68.28		6.83
CD(0.05) for interaction		NS	2.062	96.56		9.66

*Average of three replications

Mean value

		Time for mushroom production	Number of sporophores	Yield	Biological efficiency
Distance (cm)	5.00	35.75	3.25	120.83	12.08
	10.00	35.13	6.92	353.75	35.38
Period (min)	10	35.00	4.50	180.00	18.00
	15	35.00	5.17	270.83	27.08
	20	35.75	6.67	324.17	32.42
	25	36.00	4.00	174.17	17.42

(5 cm, 25 min) produced the minimum yield of 65 g, which was on par with T_2 (5 cm, 15 min), T_3 (5 cm, 20 min) and T_1 (5 cm, 10 min). T_5 (10 cm, 10 min) and T_8 (10cm, 25 min) produced yield which were on par. There was yield increase in the treatments T_6 (10cm, 15 min) and T_7 (10 cm, 20 min) of about two times that of T_8. The control which produced maximum yield of 521.66 g was on par with the treatment T_7 having a yield of 496.67 g and T_6 with 433.33 g (Table 22, Fig. 23 and Plate 19).

Since the yield obtained from irradiated cultures were less than control percentage increase over control was negative. The maximum reduction in yield was obtained in T_4 (-87.86 per cent) and minimum yield reduction in T_7 (-4.34 per cent).

4.9.1.1.2.4 Biological Efficiency (Per cent)

The biological efficiency of the non irradiated culture was highest with 52.1 per cent. Irradiation of cultures with UV rays at 10 cm from the source for different periods (10, 15, 20, and 25 min) gave higher biological efficiencies of 20.17, 43.33, 49.67, and 28.33 per cent respectively. Lower biological efficiencies were observed when the irradiation of culture was done with UV ray at 5 cm from source for 10, 15, 20 and 25 minutes *i.e.*, 15.83, 10.83, 15.17 and 6.5 per cent respectively (Table 22).

4.9.1.1.3 Estimation of Nutrients /Proximate Constituents

4.9.1.1.3.1 Moisture Content and Dry Matter

There was no significant difference between the irradiated cultures and non-irradiated cultures with respect of moisture content. It was observed that control had maximum amount of moisture content of 92.65 per cent. The irradiation of cultures 5 cm away from source for 10, 15, 20, and 25 minutes showed decrease in moisture content with increase in time of exposure *i.e.*, 92.58, 91.39, 89.66and 90.04 per cent. But the moisture content of the culture exposed to UV for 25 minutes was 90.04 per cent. Irradiation of culture 10 cm away from source for different period 10, 15,

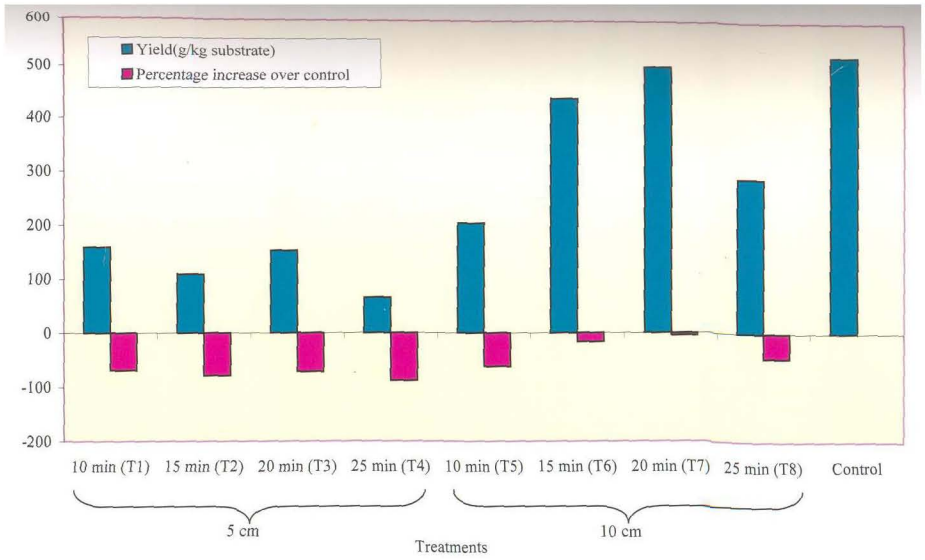


Fig. 23 Efficacy of UV irradiation on the yield of *Calocybe*

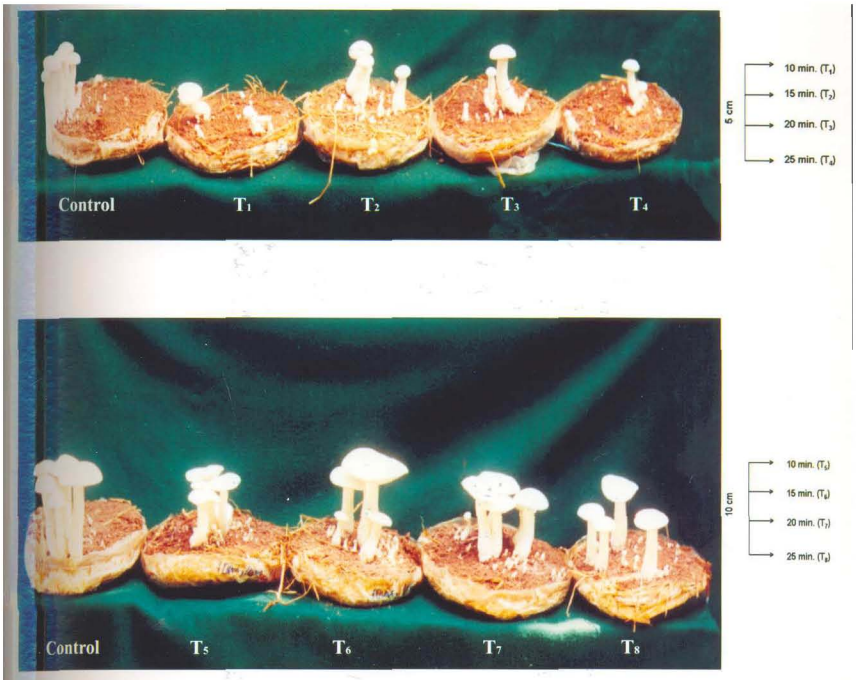


Plate 19 Effect of UV irradiation on the yield of *Calocybe*

20 and 25 minutes initially there was a decline followed by an increase in moisture content (Table 23).

The dry matter content of non-irradiated control was much lower than the irradiated ones (7.35 per cent). The irradiation of cultures 5 cm away from source for 10, 15, 20 and 25 minutes showed an increase in dry weight up to 20 minutes followed by a decline 7.41, 8.61, 10.34 and 9.66 per cent respectively (Fig. 24).

The cultures irradiated 10 cm from away from UV source for different period initially showed an increase followed by a decline (Table 23).

4.9.1.1.3.2 Total Soluble Solids

The TSS content was estimated as brix using refractometer. There irradiation of cultures did not bring any increase in TSS when compared with the control. In most cases value was 5 (Table 23).

4.9.1.1.3.3 Fibre Content

The analysis of fibre content in UV irradiated cultures indicated an increasing trend from T_1, T_2, T_3, and T_4 and in the case of irradiation of UV 10 cm from source there was an increasing trend followed by a decline at 25 min. The fibre content of non- irradiated control was 10.50 per cent which was on par with T_5 and T_6 (Table 23).

It was observed that irradiation with T_6 and T_2 had similar fibre content of 10.75 per cent and 10.96. Similar results were also obtained with T_1 and T_5 (Fig. 24).

4.9.1.1.3.4 Protein Content

The protein content in the irradiated and non-irradiated cultures were estimated as µg of protein/ g. It was observed that in control it 44 µg whereas in T_1, T_2, T_3 and T_4 (5 cm distance source) there was an increasing trend from 42 µg, 48 µg, 50 µg and 58 µg respectively. The same trend was also noticed in the irradiation of culture 10 cm away from source for

Table 23 Nutrient content in UV irradiated mushrooms

Treatment		Moisture content (%)*	Dry wt (%)*	Total soluble solids (brix)*	Fibre content (%)*	Protein (µg/g)
5 cm	10 min (T_1)	92.58	7.42	5	9.07	42
	15 min(T_2)	91.39	8.61	5	10.96	48
	20 min(T_3)	89.66	10.34	5	13.79	50
	25 min(T_4)	90.04	9.66	5	15.35	58
10 cm	10 min (T_5)	91.72	8.28	5	9.79	52
	15 min (T_6)	89.34	10.66	5	10.75	55
	20 min (T_7)	89.81	10.19	5	14.11	64
	25 min (T_8)	91.33	8.67	5	13.09	69
Control		92.65	7.35	5	10.50	44
CD(0.05) for distance		9.66	0.89		0.26	
CD(0.05) for period		13.66	1.35		0.63	
CD(0.05) for interaction		19.32	2.46		1.42	

*Average of three replications

Mean value

		Moisture content	Dry wt	Fibre content
Distance (cm)	5.00	90.99	9.01	12.29
	10.00	90.54	9.45	11.93
Period (min)	10	92.14	7.85	9.43
	15	90.36	9.63	10.86
	20	89.73	10.27	13.95
	25	90.83	9.17	14.22

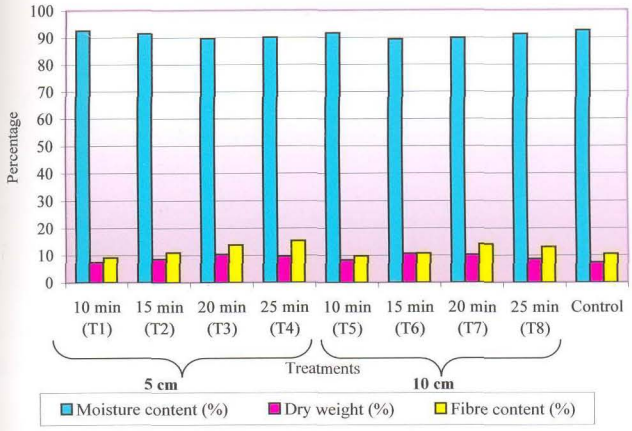


Fig. 24 Nutrient status of UV irradiated cultures

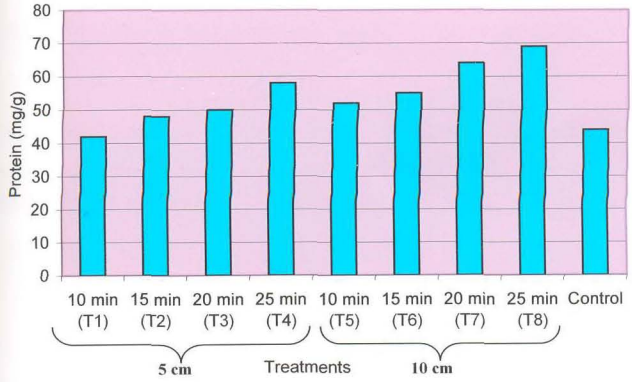


Fig. 25 Protein content in UV irradiated cultures

different period 52 µg, 55 µg, 64 µg and 69 µg respectively (Table 23 and Fig. 25).

4.9.1.1.4 Keeping Quality

Keeping quality of irradiated and non-irradiated cultures did not vary widely. Irradiation of culture at 5 cm distance 10,15, 20, and 25 minutes were on par with keeping quality for 16.00, 16.50, 18.50 and 16.50 days respectively. Control culture had a shelf life of 21.5 days. The treatments T_5, T_6, T_7 and T_8 had a shelf life of 18.00, 21.50, 21.50 and 20. 50 days and inferior one was T_1 treatment.

There was no significant difference between the irradiated and non-irradiated culture, with respect to the keeping quality under room temperature. Under room temperature the irradiated cultures could be stored upto 4 days compared to three days in control. Irradiation for 15, 20, 25 minutes from different distances had the same shelf life, indicating the in effective nature (Table 24 and Fig. 26).

4.9.1.2 Irradiation with Gamma (γ) Rays

The selected isolate of *Calocybe* were subjected for γ irradiation studies. Gamma irradiation was done at five different levels 0.5 KR, 1 KR, 1.5 KR, 2 KR and 2.5 KR. The irradiated cultures were maintained in PDA slants by periodical subculturing.

4.9.1.2.1 Radial Growth of γ Irradiated Cultures

The cultures irradiated at different levels were tested for the mycelial proliferation and radial growth. It was found that culture irradiated at 2.5 KR showed maximum mycelial growth of 5.7 cm which was on par with that of 2 KR *i.e.*, 5.6 cm and control. The increase in irradiation dose enhanced the mycelial growth of native isolate of *Calocybe*. There was no significant difference with respect to the growth of irradiated and control in 14 days as all these cultures completed 9cm growth in two weeks time (Fig. 27 and Plate 20).

Table 24 Shelf life of mushrooms of UV irradiated cultures

Treatment		Keeping quality (days)*	
		Room temp	Refrigerator
5 cm	10 min (T_1)	2.50	16.00
	15 min(T_2)	3.00	16.50
	20 min(T_3)	3.5	18.50
	25 min(T_4)	4.00	16.50
10 cm	10 min (T_5)	2.50	18.00
	15 min (T_6)	4.00	21.00
	20 min (T_7)	3.50	21.50
	25 min (T_8)	3.00	20.50
Control		5.00	21.50
CD(0.05) for distance		NS	NS
CD(0.05) for period		NS	NS
CD(0.05) for interaction		0.852	3.152

*Average of two replications

		Keeping quality	
		Room temperature	Refrigerator
Distance (cm)	5.00	3.25	16.88
	10.00	3.25	20.55
Period (min)	10	2.50	17.00
	15	3.50	18.75
	20	3.50	20.00
	25	3.50	18.50

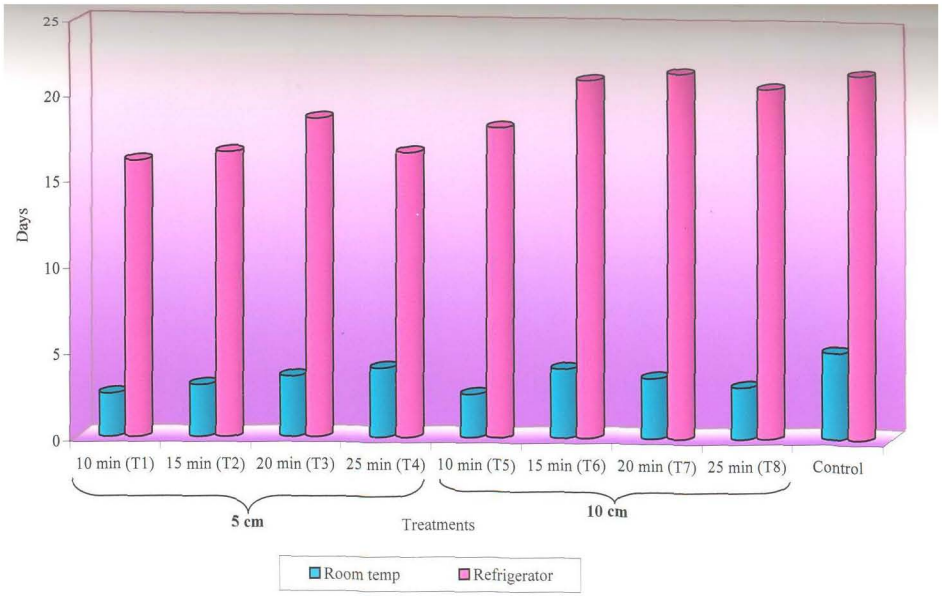


Fig. 26 Shelf life of UV irradiated *Calocybe*

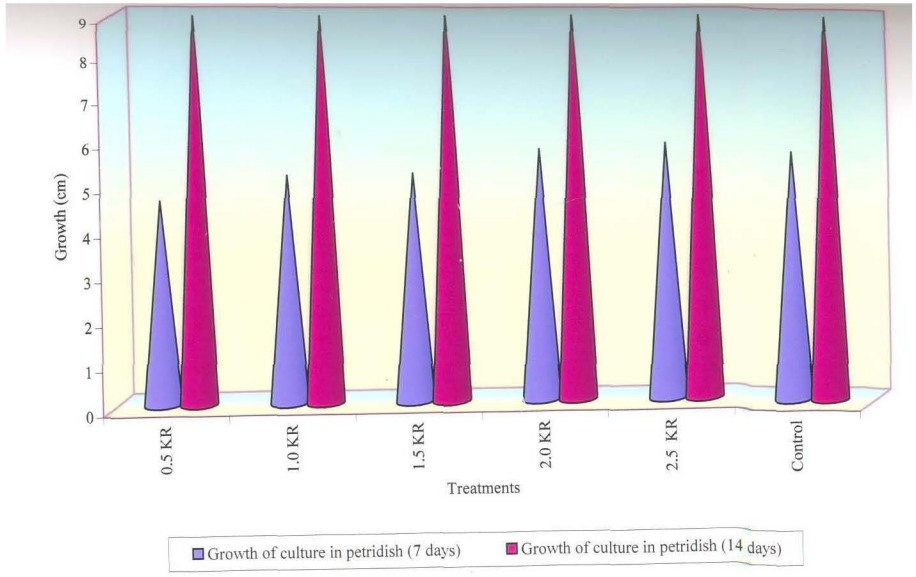
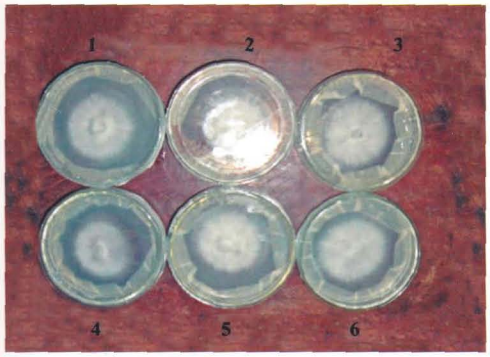


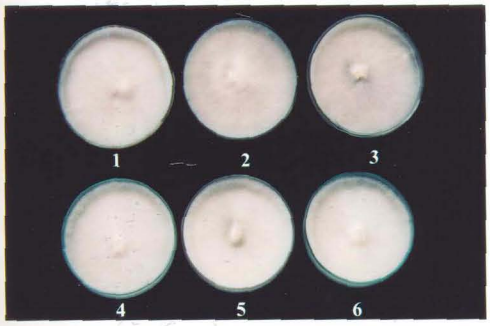
Fig. 27 Effect of gamma irradiation on the growth of *Calocybe*



Growth after seven days

Growth after 14 days

1. Control
2. 0.5 KR
3. 1.0 KR
4. 1.5 KR
5. 2.0 KR
6. 2.5 KR



Nature of growth

Plate 20 Effect of gamma irradiation on the growth of *Calocybe*

Compared with control which had better fluffy mycelial growth irradiation had a depressive effect on the growth of mycelium. It was observed that with increase in the radiation dose there was increase in fluffy nature of mycelial growth but never reached with that of non- irradiated control (Table 25 and Plate 20).

4.9.1.2.2 Evaluation of Yield Performance of γ Irradiated Cultures

4.9.1.2.2.1 Time for Mushroom Production (Table 26)

The time taken for mushroom production varied widely with irradiation dose. The minimum period for mushroom production was in non-irradiated control (29.66 days). The irradiation at 0.5 KR, 1.0 KR, and 1.5 KR which were on par took 34.33, 35.66, and 36.00 days respectively. The higher levels of irradiation had an inhibitory effect on mushroom production, hence took 37.33, and 38.33 days respectively (Fig. 28).

4.9.1.2.2.2 Number of Sporophores Harvested

The yield performance of irradiated cultures at different exposure levels were compared with the control, a non-irradiated parent culture. It was found that the culture irradiated at 1.5 KR was most efficient in production of maximum number of sporophores (12.67) and maximum yield of 481.67 g followed by 1.0 KR level. The culture irradiated at 2 KR was found to be statistically on par with 1.0 KR and also with 2.5 KR control and 0.5 KR. Very low fruiting body formation was observed at 0.5 KR and lowest yield was recorded in 2.5 KR (Table 26).

4.9.1.2.2.3 Yield

As the irradiation dose increased from 0.5 KR to 1.5 KR there was an increase in the yield. The irradiating doses above 1.5 KR showed a decreasing trend in the yield etc. Maximum yield of 481.67g was noticed in culture irradiated at 1.5 KR. Irradiation at 0.5KR and 2.5KR produced yield much less than the non irradiated ones (Table 26 and Fig. 29).

Table 25 Effect of gamma irradiation on the growth of *Calocybe*

Sl. No	Treatments	Growth of culture in petridish(cm) (7 days)*	Nature of mycelial growth
1.	T_1 – 0.5 KR	4.57	++++
2.	T_2- 1.0 KR	5.10	+++
3.	T_3- 1.5 KR	5.10	++++
4.	T_4- 2.0 KR	5.60	++++
5.	T_5- 2.5 KR	5.70	++++
6.	T_6- Un irradiated control	5.53	+++++
	CD(0.05)	0.564	

*Average of three replications

Table 26 Effect of gamma irradiation on the yield of *Calocybe*

Sl No	Treatment	Time taken for mushroom production(days)*	No of sporophores harvested*	Yield (g/kg substrate)*	Percentage increase over control	Biological efficiency(%)*
1.	T_1 – 0.5 KR	34.33	3.33	248.33	-23.07	24.83
2.	T_2 – 1.0 KR	35.67	6.67	325.00	6.90	32.50
3.	T_3 – 1.5 KR	36.00	12.67	481.67	56.25	48.17
4.	T_4 – 2.0 KR	37.33	5.33	343.33	9.32	34.33
5.	T_5 – 2.5 KR	38.33	4.66	245.00	-17.83	24.50
6.	T_6- Un irradiated control	29.66	4.66	320.00		32.00
	CD(0.05)	2.138	2.905	106.501	50.999	10.650

*Average of three replications

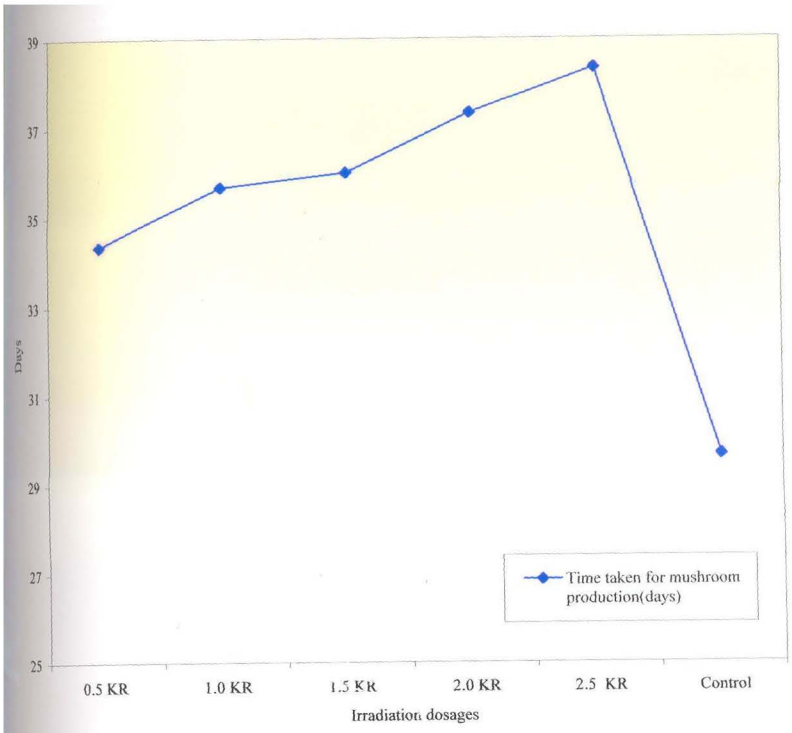


Fig. 28 Effect of gamma irradiation on the initiation of mushroom primordia

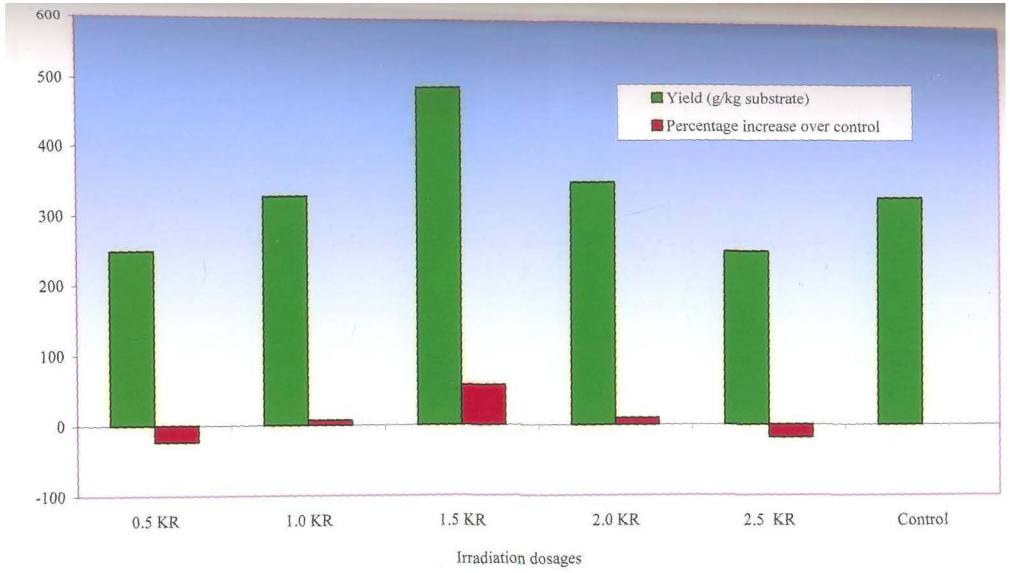


Fig. 29 Effect of gamma irradiation on the yield of *Calocybe*

Lower and higher irradiation dosages resulted in reduced yield when compared to control hence percentage increase over control was negative. Irradiation at 1.0, 1.5, and 2.0 KR resulted in 6.90, 56.25 and 9.32 percentage increase over control (Plate 21).

4.9.1.2.2.4 Biological Efficiency (Per cent)

Biological efficiency was maximum with irradiation at 1.5 KR level and minimum with 2.5 KR level. The biological efficiency also showed an increasing trend upto 1.5 KR followed by a decline at higher irradiation dosage. The control treatment produced biological efficiency higher than the lowest and highest irradiation dosage (Table 26).

4.9.1.2.3 Estimation of Nutrients

4.9.1.2.3.1 Moisture Content and Dry Matter

The moisture content per cent was observed to be maximum with control (91.82). Irradiation level from 0.5, 1.0, 1.5, 2.0 and 2.5 KR has a decreasing trend with 89.48, 88.82, 84.81, 84.74 and 81.29 per cent moisture. There was significant difference between the treatments control and 0.5 KR, 1.0 KR and 1.5 KR, 2 KR and 2.5 KR (Table 27 and Fig. 30).

In contrast to the moisture content dry weight had an increasing trend from 0.5, 1.0, 1.5, 2.0 and 2.5 KR with 10.52, 11.18, 15.19, 15.26 and 18.71 per cent respectively. The maximum dry weight was observed in 2.5 KR irradiated culture and minimum with control (8.18 %).

4.9.1.2.3.2 Total Soluble Solids

The studies on total soluble solids of γ irradiated and non-irradiated cultures reveals that there is no significant difference between these treatment. TSS was 5-6 brix in irradiated cultures (Table 27).

4.9.1.2.3.3 Fibre Content

The fibre content in the control was 9.07 per cent which was on par with the fibre content 0.5 KR and 1 KR (8.8 and 9.8 per cent). The

Table 27 Effect of gamma irradiation on the nutrient composition of mushroom

Sl No	Treatment	Moisture content (%)*	Dry weight (%)*	Total soluble solids (brix)	Fibre content (%)*	Protein content (µg/g)
1.	T_1 – 0.5 KR	89.48	10.52	5	8.81	68
2.	T_2- 1.0 KR	88.82	11.18	5	9.83	118
3.	T_3- 1.5 KR	84.81	15.19	6	13.05	86
4.	T_4- 2.0 KR	84.74	15.26	5	11.54	72
5.	T_5- 2.5 KR	81.29	18.71	5	11.22	70
6.	T_6- Un irradiated control	91.82	8.18	6	9.07	37
	CD (0.05)	1.184	1.183		1.767	

*Average of three replications

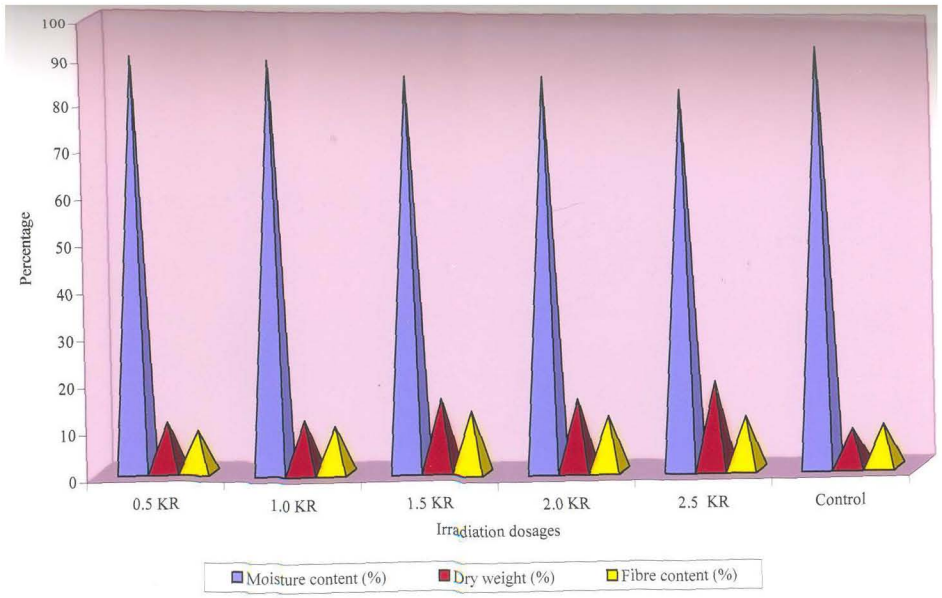


Fig. 30 Effect of gamma irradiation on the proximate nutrient content of *Calocybe*

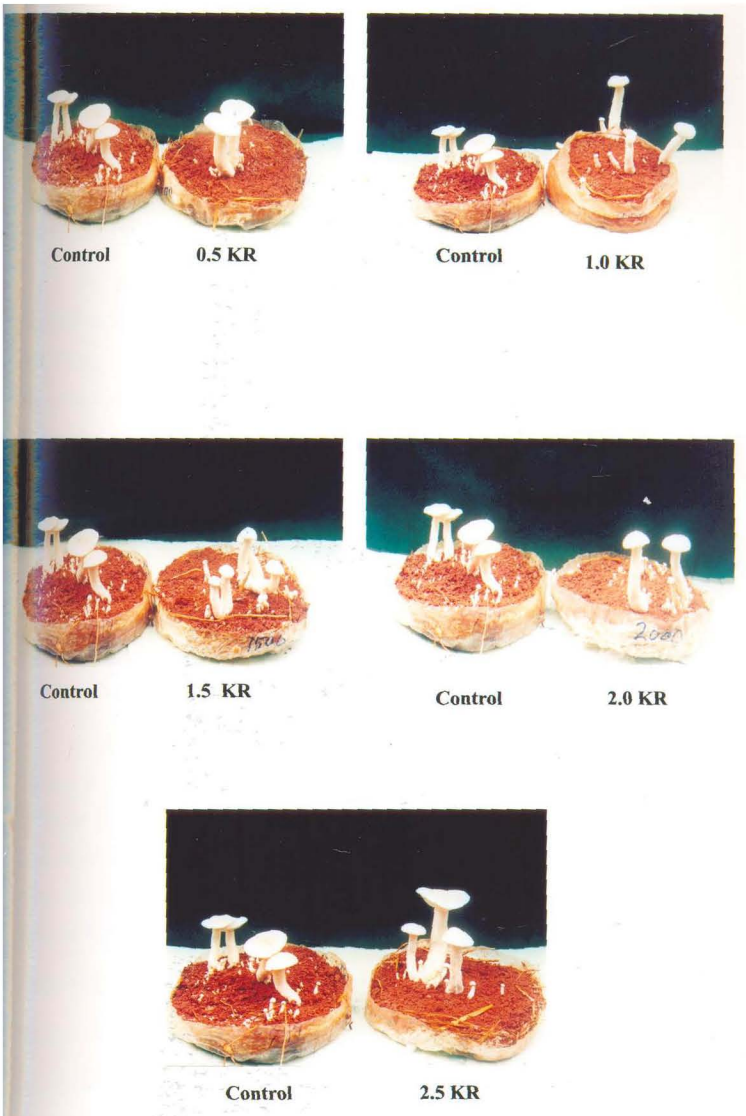


Plate 21 Effect of gamma irradiation on the yield of *Calocybe*

maximum fibre content of 13.05 per cent was noticed when cultures were irradiated at 1.5 KR. The irradiation dosage 2 KR and 2.5 KR showed reduced fibre content of 11.54 and 11.22 per cent respectively. It was noticed that increase irradiation from 0.5 KR to 1.5 KR enhanced fibre content but from 1.5 to 2.5 KR there was a decline (Table 27 and Fig. 30).

4.9.1.2.3.4 Protein Content

The protein content of mushrooms was observed to be raised by irradiation. It was observed that protein content in non-irradiated control was 37 µg. The increase in irradiation dosage from 0.5 KR to 1.0 KR had an increasing effect with 68 - 118 µg respectively. But the irradiation with higher dosage (1.5 KR, 2.0. KR and 2.5 KR) showed a decreasing trend from 86 - 70 µg (Table 27 and Fig. 31).

4.9.1.2.4 Keeping Quality

The shelf life study indicated that there was enhancement in keeping quality of irradiated culture than non irradiated one at room temperature. The keeping quality enhanced from 0.5 KR – 2.5 KR with the days 5.67, 5.66, 6.33, 9.33, 9.66 days whereas in control it was 3.33 days.

Under refrigerated condition also there was enhanced shelf life of irradiated mushrooms than control. Maximum shelf life of 37.33 days was observed in cultures irradiated at 2 KR which was on par with 2.5 KR. The least shelf life was observed in control with 21.67 days. The shelf life of other irradiated cultures were 30.33, 30.67, and 30.67 days respectively with 1.5 KR, 1.0 KR, and 0.5 KR (Table 28, Fig. 32 and Plate 22).

4.9.2 Mating/Crossing of Isolates of Dissimilar Characters

4.9.2.1 Multispore Crossing

The crossing of multispore cultures was done as described in 3.7.2.1. The study revealed that in most of the isolates there was no crossing. The hyphae of the two dissimilar isolates instead of fusing either diverged or

Table 28 Shelf life of mushrooms of gamma irradiated cultures

Sl No	Treatments	Keeping quality (days)*	
		Room temp	Refrigerator
1.	T_1 – 0.5 KR	5.66	30.67
2.	T_2- 1.0 KR	5.66	30.67
3.	T_3- 1.5 KR	6.33	30.33
4.	T_4- 2.0 KR	9.33	37.33
5.	T_5- 2.5 KR	9.66	35.33
6.	T_6- Un irradiated control	3.33	21.67
	CD (0.05)	1.624	2.516

*Average of three replications

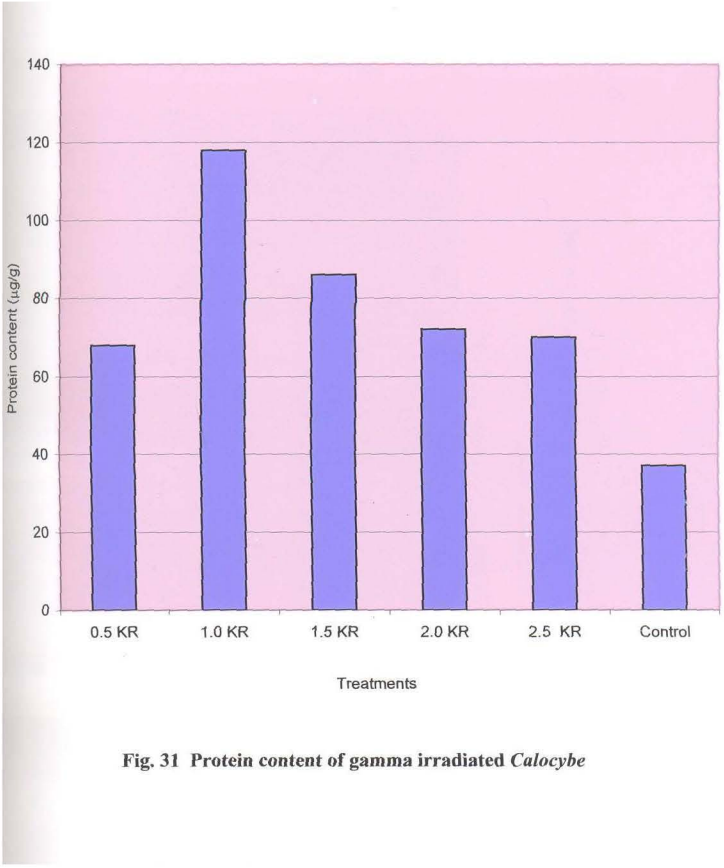


Fig. 31 Protein content of gamma irradiated *Calocybe*

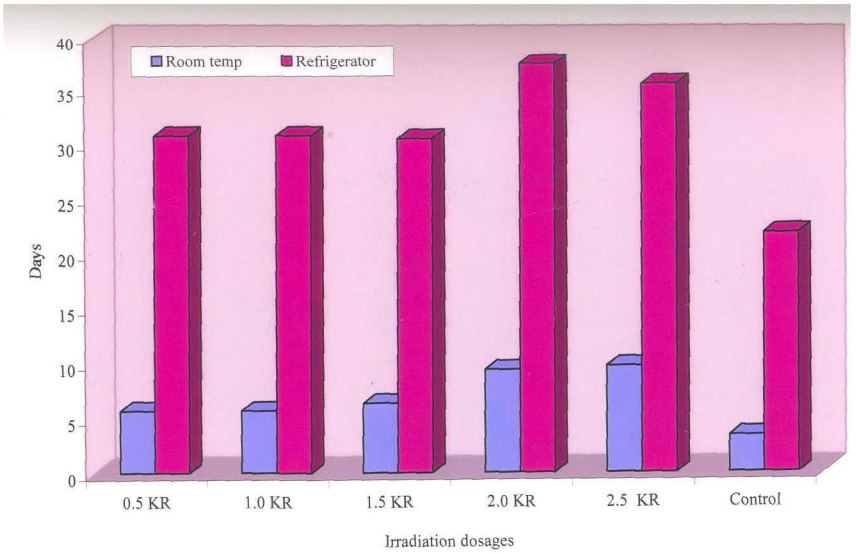


Fig. 32 Effect of gamma irradiation on the shelf life of *Calocybe*

1. Control
2. 0.5 KR
3. 1.0 KR
4. 1.5 KR
5. 2.0 KR
6. 2.5 KR

Plate 22 Shelf life of mushrooms of gamma irradiated cultures

overgrown. In these crossings, the development of thick strands of mycelium was absent which indicated mating incompatibility.

4.9.2.2 Isolation of Single Spore

The isolation of single spores of different isolates of *Calocybe* with dissimilar characters were done using serial dilution as described under 3.8.2.2. The single spores were marked with a pen under a dissection microscope. The single spores were transferred to PDA using an inoculation needle. The single spores were maintained on PDA slants with periodical subculturing.

4.9.2.3 Hybridisation of Single Spore Isolate

The hybridization of single spore isolates was done by dual culture technique as described in 3.8.2.3. The mycelium of selected isolates grew and at the point of interception, the development of thick strand of mycelium indicated hybridization between isolates of dissimilar characters.

Several crosses were attempted with selected isolates of *Calocybe*. The single spore identified as A_1, A_2, A_3, B_1, B_2 were crossed as $A_1 \times B_1$, $A_1 \times B_2$, $A_2 \times B_1$, $A_2 \times B_2$, $A_2 \times B_1$, $A_2 \times B_2$, $A_3 \times B_1$, $A_3 \times B_2$. Of these crosses, $A_1 \times B_2$, $A_2 \times B_1$, $A_2 \times B_2$ were found to be incompatible. The crosses $A_1 \times B_1$, $A_3 \times B_1$ and $A_3 \times B_2$ were selected. On further studies crossing between $A_3 \times B_2$ was persistent so it was selected (Plate 23).

The hybrid culture was transferred to processed paddy grains for spawn production. This spawn was used for bed preparation.

4.9.2.3.1 Number of Sporophores Harvested and Yield

The study conducted to evaluate the yield of parents and their crosses revealed, that the hybrid produced more number of sporophores (12.59) when compared to both parents, which yield 4.74 and 6.76 numbers respectively. Similarly, increased yield was also noticed in the case of the hybrid. The hybrid produced higher yield of 548.81 g, where as parent 1,

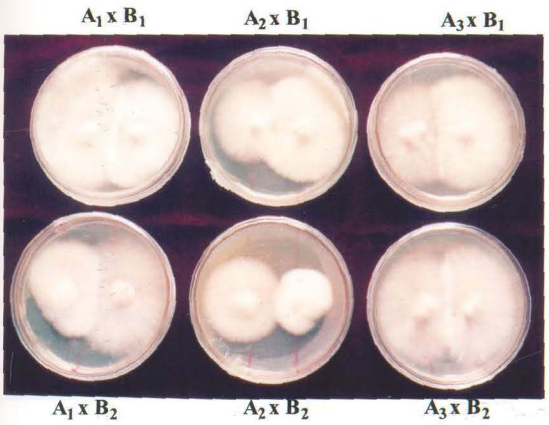


Plate 23 Hybridisation of cultures

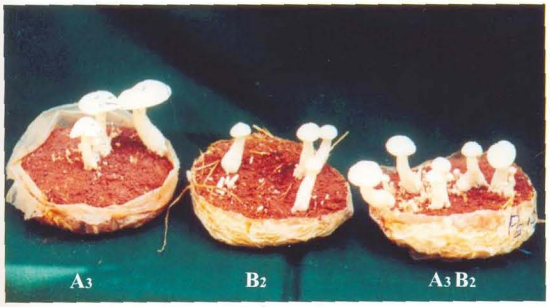


Plate 24 Performance of parents and hybrid

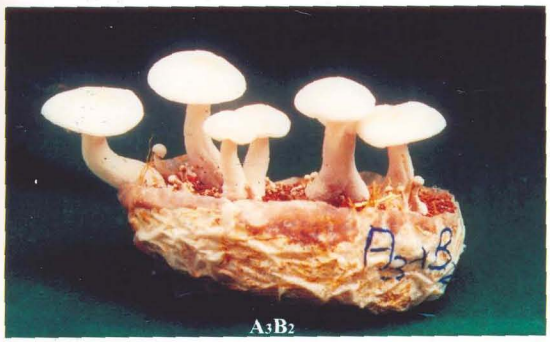


Plate 25 Hybrid

produced 335.61 g and parent 2 produced 300.54 g (Table 29, Fig. 33 and Plates 24 and 25).

Biological efficiency of the hybrid was higher when compared to both the parents which were on par (Table 29).

4.9.2.3.2 Estimation of Nutrients in Hybrids (Table 30)

4.9.2.3.2.1 Moisture Content and Dry Weight

There was significant difference between the hybrids and their parents with respect to the moisture content. The maximum moisture content was noticed in parent 1 (A_3) 93.84, followed by parent 2 (B_2) 92.19 per cent and hybrid with 91.03 per cent.

Higher the moisture content, lesser will be the dry weight. The dry weight also varied significantly between the parents and hybrid. In contrast to the moisture, the maximum weight was observed in the hybrid (8.96 per cent) followed by parent 2 (B_2) 7.81 per cent and parent 1 (A_3) 6.16 per cent (Table 30 and Fig. 34).

4.9.2.3.2.2 Total Soluble Solids

The total soluble solids content did not vary significantly between the parents and the hybrid. The hybrid had TSS of 5, whereas A_3 had seven and B_2 had six brix.

4.9.2.3.2.3 Fibre Content

The fibre content of the hybrid mushroom (13.59) was on par with the parent A3 (14.81). But there was significant difference in fibre content between the two parents. The parent A_3 had 14.81 per cent and B_2 had 17.33 per cent (Table 30 and Fig. 34).

4.9.2.3.2.4 Protein Content

The protein content derived from the standard graph using Bradford method showed enhanced amount in the hybrid 37 µg when compared to both the parents having 23 µg, 28 µg respectively (Fig. 35).

Table 29 Yield characters of the parents and hybrid

Sl No	Name of the isolate	No of sporophores harvested*	Yield (g/kg substrate)*	Biological efficiency (%)*
1.	A3 (P1)	4.74	335.61 (18.31)	33.56 (5.79)
2.	B2 (P2)	6.76	300.54 (17.33)	30.05 (5.48)
3.	A3B2 (Hybrid)	12.59	548.81 (23.42)	54.88 (7.41)
	CD(0.05)	0.286	0.6475	0.631

*Average of five replications

Table 30 Nutrient composition of parents and hybrid

Sl No	Name of isolate	Moisture content (%)*	Dry wt (%)*	Total soluble solids (brix)	Fibre content (%)*	Protein (µg/g)
1.	A3 (P1)	93.84	6.16	7	14.81	23
2.	B2 (P2)	92.19	7.81	6	17.33	28
3.	A3B2 (Hybrid)	91.03	8.97	5	13.59	37
	CD(0.05)	0.825	1.060		1.592	

*Average of five replications

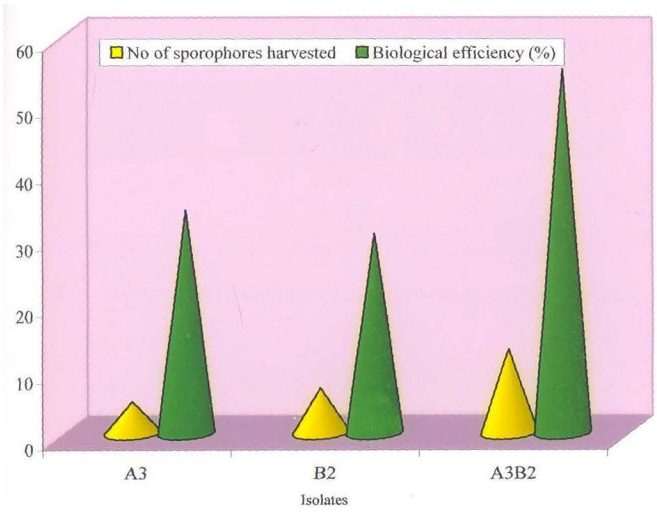


Fig. 33 Yield characters of hybrid compared to parents

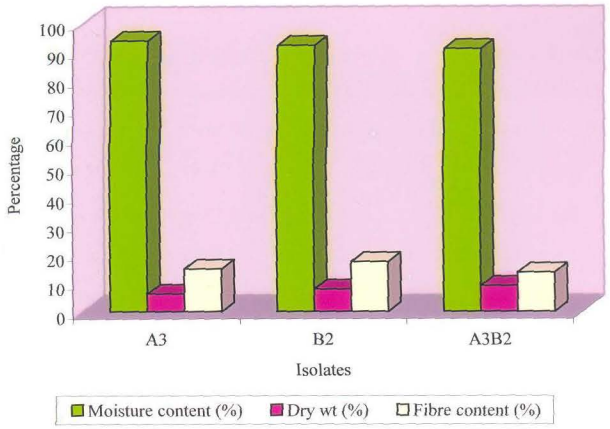


Fig. 34 Proximate constituents of hybrid compared to parents

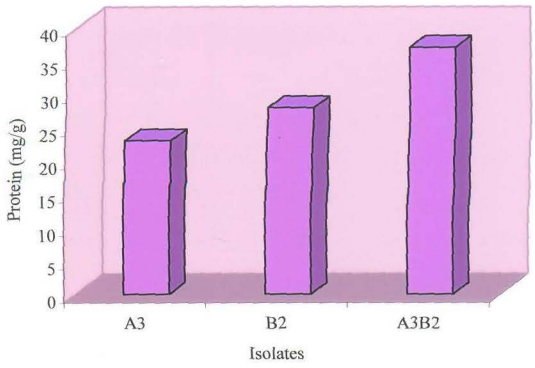


Fig. 35 Protein content of hybrid compared to parents

4.9.4 ENZYME ACTIVITY

4.9.4.1 Phenyl alanine ammonia lyase (PAL)

The studies on the changes in PAL activity was carried out to elucidate effect on the hybrid and parents. The PAL activity of the parents and hybrid was expressed μ moles of cinnamic acid equivalent/g of fresh sample/minute as shown in Table 31 and Fig. 36.

The observations showed that hybrid had 0.34 μg cinnamic acid per min per g of PAL activity which was more than that of the two parents (0.25 μg cinnamic acid per min per g and 0.08 μg cinnamic acid per min per g).

4.9.4.2 Peroxidase (PO)

The studies of the peroxidase activity were measured as change in absorbance per minute per g of mushroom sample. The activity of peroxidase was higher in the case of hybrid (0.60) compared to that of the parent (0. 50 and 0. 50) (Table 31 and Fig. 36).

4.9.4.3 Poly phenol oxidase (PPO)

The polyphenol oxidase activity was measured as change in absorbance per minute per gram of the mushroom sample. There was no significant change in the activity with respect to the parents and hybrid (Table 31 and Fig. 36).

Since the enzyme activity of hybrid and parents were very low the isozyme analysis was a failure without any result.

4.10 MOLECULAR CHARACTERIZATION

4.10.1 Molecular Characterization Using Isozyme Analysis

Molecular characterization of the hybrids and parents by the isozyme PAL, peroxidase and poly phenol oxidase was done using a native poly acrylamide gel. The procedure was done as described in 3.9.1. The result obtained was negative and no bands developed in any of the enzyme. Since enzyme activity of the mushroom was low, it could not be detected on a gel.

Table 31 Enzyme activity of parents and hybrid

Sl No	Name of isolate	PAL activity(µg of cinnamic acid/min/g)	Peroxidase activity (change in absorbance /min /g)	Poly phenol oxidase activity (change in absorbance /min /g)
1.	A3 (P1)	0.25	0.50	0.20
2.	B2 (P2)	0.08	0.50	0.00
3.	A3B2 (Hybrid)	0.34	0.60	0.03

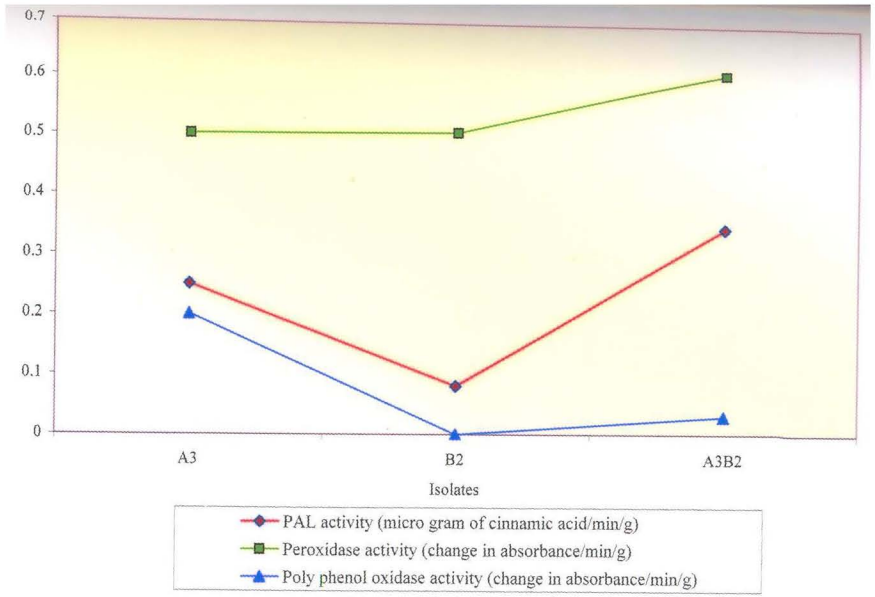


Fig. 36 Enzyme activity of parents and their hybrid

4.10.2 RAPD Analysis

4.10.2.1 DNA Extraction and Purification

DNA of the parents (A_3 and B_2) and hybrids (A_3B_2) were isolated by CTAB method. The DNA pellets after purification with RNA are treatment was resuspended in 100 µl of TE buffer. The purified DNA was electrophorised in 0.7 per cent agarose gel to assess its concentration and integrity. The purity of the samples was checked using UV/VIS spectrophotometer. The ratio of OD 260: OD 280 was 1.4 for A_3 1.7 for B_2 and 1.6 for A_3B_2. The quantity of DNA isolated from the parents and hybrid was 210 ng/µl (A_3), 540 ng/µl (B_2) and 240 ng/µl (A_3B_2) respectively.

4.10.2.2 PCR Reaction

Eight RAPD primers were screened for amplification. Five of them were selected based on their earlier research report of Singh *et al.* (2003 b). Among them only four primers *i.e.*, OPA-04, OPP-03, OPP-06 and OPP-12 produced good amplification. Primer sequence of OPA-04, OPP-03, OPP-06 and OPP-12 are as follows.

OPA-04 - AATCGGGCTG

OPP-03 - CTGATACGCC

OPP-06 - GTGGGCTGAC

OPP-12 - AAGGGCGAGT

Of these four primers the PCR amplification was reproducible with OPA-04 and OPP-12. Amplification with OPP-03 and OPP-06 was not reproducible. So finally OPA-04 and OPP-12 were selected for amplification of parents and hybrids. All the reactions were repeated thrice and produce similar results. The RAPD profile is shown in Plate 26.

Primer OPA-04 produce two bands with the DNA sample of parent A_3, three bands with parent B_2 and two bands with the hybrids. Of these one band was

Plate 26 RAPD profile

monomorphic for parent B_2 and hybrid A_3B_2. All the other bands were polymorphic.

OPP-12 produced only one band with parent A_3 and four bands for each for parent B_2 and hybrid. One band was monomorphic for both the parents and hybrids. Three bands were monomorphic for parents B_2 and the hybrid.

Score of the DNA bands is shown in Table 32.

Statistical analysis was done for the results obtained with the primers OPA-04 and OPP-12. Similarity matrix calculated on the basis of the results is shown in Table 33. The pair wise coefficient values ranged from 0.250 to 0.444. The parent A_3 and parent B_2 had 25 per cent similarity whereas the hybrid had 28.57 per cent similarity with A_3 and 44.40 per cent similarity with B_2. The matrix was subjected to unweighted pair group method analysis to develop a dendrogram (Fig. 37). The random primers distinguish the isolates into two phylogenetic groups at 0.26 similarity coefficient.

4.11 PEST

Snails occurred on mushroom beds after the pin head formation. As a result of the feeding, the stipe becomes hollow. Those mushrooms which were attacked by snails had stunted growth, discolouration and further lead to infection by saprophytic fungus like *Trichoderma* (Plate 27 and 28).

Snails could be effectively managed by spraying 0.5 per cent salt solution or by putting 5g salt on the snail if they are seen on the beds. Chemical control was not effective in the management of these pests because of the hard outer shell covering the pest.

Table 32 Scoring pattern

Parent 1 (A3)	Parent 2(B2)	Hybrid (A3B2)	Primers
0	1	0	OPA-04
1	1	0	
0	1	1	
1	0	1	
0	0	1	OPP-12
0	1	0	
0	1	1	
0	1	1	
1	1	1	

Table 33 Tree matrix

	A3	**B2**	**A3B2**
A3	1.0000000		
B2	0.2500000	1.0000000	
A3B2	0.2857143	0.4444444	1.0000000

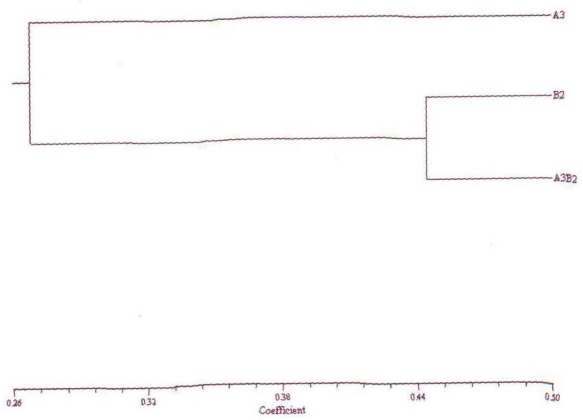


Fig. 37 Dendrogram

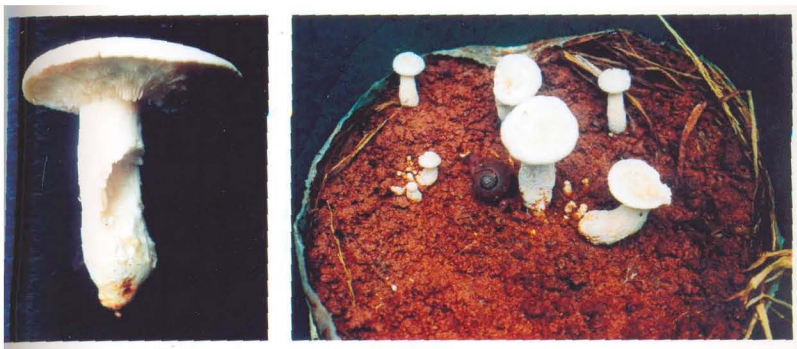


Plate 27 Nature of damage by snail

Plate 28 Snail

Discussion

5. DISCUSSION

5.1 SURVEY

Surveys were conducted in selected districts *viz.*, Thiruvananthapuram, Kollam and Pathanamthitta during monsoon and post monsoon periods to collect the native flora. As a result ten native species of *Calocybe* were collected. They were subjected to different morphological observations and brought them into pure culture. All the cultures were subjected to preliminary study to explore their suitability to bring them under cultivation. Sarkar *et al.* (1988) reported the occurrence of *Calocybe* species among the wild edible mushrooms obtained from Tripura. Gogoi *et al.* (2000) also made similar observation on the occurrence of *Calocybe indica* and *Calocybe gambosa* in the North Eastern State of Assam. Surveys were conducted by Kalpana (2003) to explore the biodiversity of milky mushroom in Tamil Nadu. Surveys conducted in Western Ghats by Anandh and Prakasam (2003) resulted in the collection of edible mushrooms like *Calocybe indica, Calocybe gambosa, Tricholoma lobayense, Tricholoma giganteum.* It was observed that organic matter rich soil, coconut tree basins were the usual spots for occurrence of *Calocybe.* This may be due to its ability to degrade lingo cellulolytic material. This is in accordance with the observations made by Krishnamoorthy (2003) who reported the presence of *Calocybe* in humus rich soil under road side trees and agricultural fields. The period of occurrence of *Calocybe* was during June to October (rainy and post rainy season). Similar observations were made by Anandh and Prakasam (2003).

5.2 IDENTIFICATION OF NATIVE ISOLATES

Mushrooms were screened for their macroscopic characters such as shape, size and colour of basidiocarp. The shape of basidiocarp was convex, later expanded and flat with whitish colour, diameter ranging from 5-25 cm. The stipe was clavate in shape with bulbous base and some times suppressed scales were

present. This is in agreement with observations made by Anandh and Prakasam (2003) on the macroscopic details of *Calocybe* sp.

5.2.1 Microchemical Reagetns

5.2.1.1 Melzers Reagent

The reaction of spore with melzers reagent resulted in non-amyloid reaction which was in accordance with the results of Anandh and Prakasam (2003).

5.2.1.2 Cotton Blue Stain

Cotton blue reaction of spores resulted in cyanophilic reaction.

5.2.2 Screening of Isolates for Organoleptic Characters

The native isolates screened for their characters like colour, appearance, texture and flavour on the basis of score card. The isolates OE-357 had maximum ranking for colour, appearance and flavour followed by the isolate OE-349 which also had better texture. These two isolates were preferred over the other isolates mainly due to its attractive nature. This is in agreement with the findings of comparative study by Balakrishnan (1994) who showed that *Pleurotus sapidus, P. membranaceous* and *P. petaloides* obtained maximum consumer acceptability with respect to colour, appearance and flavour. Overall acceptability of these species were significant when compared to the standard species *P. sajor-caju* and *P. flabellatus* which were found inferior in all the qualities. Desai *et al.* (1991) revealed that consumer acceptability of *Pleurotus sajor-caju* was poor due to the tough texture of the stipe and unattractive colour of the pileus but its flavour was found good.

5.3 ISOLATION AND PURIFICATION OF CULTURE

Tissue isolation was done and cultures were maintained in PDA slants with periodical subculturing. The isolation of *Calocybe indica* on PDA medium from the sporophore was reported by Eswaran and Susan (2003) and Theradimani *et al.* (2001).

5.4 SCREENING OF MUSHROOM ACCESSION UNDER *IN VITRO* CONDITIONS

The native isolates of *Calocybe* were screened for their growth rate indicated that there was no significant difference between the isolates with respect to seven days growth. The isolate which gave better growth in the beginning did not retain that trend later. Growth of the ten native isolates indicated that isolates OE-349, OE-357, OE-350, OE-355 and OE-356 completed growth in a fortnight. Some of the native isolates had taken more time to complete their growth in petri plates. Speedy growth of these isolates is suggestive of suitability for their cultivation. This is in accordance with the study conducted by Rafique *et al.* (1999) for the *in vitro* cultivation of *Pleurotus* species on culture media. The study revealed that *P. cornucopiae* and *P. florida* took minimum period of 10 days for full growth whereas *P. flabellatus. P. sajor-caju, P. pulmonarius* took 11 days. Maximum period was taken by *P. fossulatus* (15). It was concluded that there is variation in growth depending on the genetic make up of the species. Growth pattern of native isolates also varied from fluffy native to feable. The study indicated that the fastest growing isolates had fluffy growth where as slow growing ones had lesser growth. Rafique *et al.* (1999) proved that *P. cornucopiae, P. florida, P. flabellatus* and *P. sajor-caju* having appressed growth took only shorter period for growth whereas *P. citrinopileatus, P. pulmonarius, P. membranaceus* and *P. fossulatus* had less dense and transposed mycelial growth, since it had taken more time for full growth.

Das *et al.* (2000b) confirmed the finding of Rafique *et al.* (1999) with *P. florida, P. flabellatus* and *P. sajor-caju* having appressed growth. *P. platypus* produced less dense and transparent mycelium. Dense growth was observed in *P. ostreatus*.

Yadav *et al.* (2002) showed variation in mycelial characters of single spores of *Agaricus bisporus* with normal fluffy mycelium, strandy mycelium and normal dense mycelium. Yadav *et al.* (2003c) reported that *Agaricus bitorquis*

germplasm showed wide variation in colony morphology with strandy growth, dense growth and normal growth.

5.5 SCREENING OF MUSHROOM ACCESSIONS FOR COMMERCIAL CULTIVATION

5.5.1 Mycelial growth in Spawn

The native isolates of *Calocybe* showed wide variation with respect to the nature of mycelial growth in grains. The isolates OE-349, OE-351, OE-355 and OE-357 had fluffy growth followed by lesser fluffy nature of growth in OE-350, OE-352, OE-356 and OE-358.

Among the ten native isolates, variation was noticed with respect to the time for spawn. It was observed that OE-355 took minimum period, whereas OE-356 took maximum period. This is in accordance with results obtained by Suharban (1987) who conducted monographic study on the edible species of *Pleurotus*. The results revealed that maximum growth in 21 days was obtained in *Pleurotus opuntiae* and minimum growth in *Pleurotus florida*. Lesser time requirement for spawn growth reflects the ability of the isolate to utilise the nutrient source without any delay. Longer duration for spawn growth may be attributed to their lesser adaptability of the isolate in the new substrate rather than the natural source. The native isolates of *Calocybe* vary with respect to the genetic make up, environment requirement and original substrate from which it was obtained. This may also contribute to the variation in nature of spawn growth. Cereal grains were preferred over pulses for the preparation of spawn. The study conducted by Kumar and Chandra (1988) also showed wide variation in the nature of mycelial growth of *Agaricus* from silky dense to strandy and fluffy with different substrates and time for spawn run also varied from 23-34 days. Similar observations were made by Guleria *et al.* (1989) on the mycelial growth and time taken for spawn growth. He observed that variation was noticed in nature of mycelial growth with white silky dense growth in wheat and jowar grains having minimum period of 28 days, while fluffy growth on bajra meal and strandy growth on compost which took maximum period of 36 days for spawn growth.

5.5.2 Screening Trials

5.5.2.1 Comparative Characters of Native Isolates on Beds

The native isolates of *Calocybe* took 8 - 20 days for initial production. The isolate OE-349 took only eight days for initial appearance. Earliness in primordial initiation was a preferred criteria for the early harvesting of *Calocybe*. The earliness in primordial initiation may be due to the genetic make up of the mushroom and also the adaptability to newer substrate. Larger period taken by several isolates may be due to their inability to utilize the new lignocellulosic material. The harvesting period was about 16.67 days in case of OE-349 whereas the isolate OE-356 took 20 days for initial appearance showing longer maturity period. When the native isolates were introduced into the newer substrate may take some time for the production of enzymes for the degradation of a particular substrate. This is in accordance with the result of experiment conducted by Anandh (2001). The experiment revealed that among the native isolates obtained from survey in Western Ghats, *Calocybe indica* and *Tricholoma lobayense* took eight days for initial appearance in the beds and harvest was done on the eleventh day. Krishnamoorthy and Muthusamy (1997b) observed the initial appearance of *Calocybe* in eight to ten days after casing and took eight days for maturity. But in contrast to the above observation, Purkayastha *et al.* (1981) and Purkayastha (1985) reported appearance of pinheads of *Calocybe* in three weeks after spawning, three weeks after casing and maturity in a week time.

5.5.2.2 Comparative Yield Performance of Native Isolates

The mortality percentage of native isolates varied from 0 – 90 per cent. Lowest mortality percentage was observed in the isolate OE-355 as there was only a single sporophore developed, which was harvested. Among the other isolates, OE-349 had the lowest mortality percentage. Though the isolate OE-355 had low mortality percentage, it is not a preferred strain as it yielded only a single sporophore from a bed. Maximum number of sporophores were harvested from the bed with lowest mortality percentage.

Yield of native isolates of *Calocybe* also showed considerable variation. The yield varied from 51.66 – 368.33 g/kg among different isolates. The maximum biological efficiency among different isolates was noticed in OE-349 (36.80) per cent followed by OE-357, OE-350and OE-351. Higher biological efficiencies of 140-145 per cent was reported by Krishnamoorthy and Muthusamy (1997 b), Theradimani *et al.* (2001) and Krishnamoorthy (2003). Lower biological efficiency of 85 per cent was obtained in spent mushroom substrate (Balakrishnan and Das, 2001). The result of the experiment on yield performance of native isolates was in confirmation with low biological efficiency yield of 500 g/4 kg substrate in *Calocybe* cultivation (Purkayastha, 1985). Anandh (2001) reported the biological efficiencies of various edible mushrooms *Calocybe indica* with 56 per cent, *Calocybe gambosa* with 61.67 per cent and *Tricholoma* with 82 per cent.

5.5.2.3 Comparative Morphology of Native Isolates of Calocybe

The characters of stipe and pileus of native isolates showed that the stipe length varied from 5.00 – 10.83cm, stipe diameter 4.40 – 8.93 cm and pileus diameter 5.50 – 9.73cm

The results were in accordance with observations of Pandey and Tewari (2003) who reported characters of *Calocybe* namely stipe length of 8.0 – 13.2 cm, stipe diameter of 4.7 – 13.8 cm and pileus diameter of 6.2 – 10.3 cm. Similar observations were also made by Krishnamoorthy *et al.* (2005 b) with stipe length ranging from 6 – 10 cm, diameter of pileus from 6.8 – 8.6 cm in the case of *Calocybe indica* and *Tricholoma* sp.

The average weight of a single sporocarp of native isolates on beds ranged from 10 – 70 g. Similar observation was also reported by Krishnamoorthy (2003) who noted average weight of a single fruiting body ranged from 5 – 60 g. Studies of Pandey and Tewari (2003) and Krishnamoorthy *et al.* (2005 b) also revealed, weight of single fruiting body of *Calocybe* as 56.51g and 34.7 – 46.8 g respectively.

5.5.3 Screening of Post Harvest Quality

The shelf life of native isolates of *Calocybe* under room temperature and refrigeration showed that mushrooms when stored in non perforated polypropylene covers had better keeping quality than in polypropylene covers with perforation. The native isolates of *Calocybe* had better shelf life of 12.67 – 24.67 days when stored in refrigerated condition. The results were in agreement with the findings of Dhar (1992) who stated better shelf life of mushrooms in non perforated polyethylene packs at low temperature when compared to perforated packs at high temperature. The results were also in agreement with findings of Saxena and Rai (1988) that *Agaricus* could be stored in non polyethylene bags for four days at 5°C, two days at 10°C, and one day at 15 °C. Mehta and Jandaik (1989) also made similar observation in *Pleurotus sapidus*. Mushrooms even after harvest, under goes post harvest changes including respiration, senescence which leads to browning.

Better shelf life of *Calocybe* is attributed to the reduced activity of enzyme at low temperature. There is a slow down of maturity and conservation of metabolites to maintain the cell functions due to low temperature. Perforations provide greater vent area which allows more access of oxygen which directly increases the respiration rate. Respiration in a closed system (non perforated) oxygen is deplected with concomitant accumulation of carbon dioxide resulting in reduced rate of respiration until an equilibrium is reached (Saxena and Rai, 1988).

5.6 NUTRIENT CONTENT / PROXIMATE CONSTITUENTS

The moisture content of native isolates of *Calocybe* ranged between 80.92 – 89.64 per cent with maximum content in isolate OE-351 followed by OE-349 and minimum in OE-355. In contrast to moisture content, dry weight showed a reverse trend with maximum dry weight in isolate OE-355 having high moisture content. The dry weight of isolate OE-351 was the least.

The fibre content of the various isolates ranged from 16.02 to 24.1 per cent. The maximum fibre content was noticed in isolate OE-356 and minimum in OE-349. The protein content of *Calocybe* isolates varied from

28 – 42 µg /g with maximum in OE-349 42µg /g and minimum on OE-356. The total carbohydrates ranged from 8.33 – 20.67 µg/g. These results were in agreement of the finding of Anandh (2001). The findings revealed the nutrient composition of *Calocybe indica* with 88.37 per cent moisture, 11.63 per cent dry matter, 26.5 per cent protein, 36.5 per cent fibre, carbohydrate content of 8.80 per cent and *Tricholoma lobayanese* with 85.20 per cent moisture, 14.80 per cent dry matter, 33.2 per cent protein, 23.74 per cent fibre and 11.38 per cent carbohydrate. Krishnamoorthy and Muthusamy (1997 a) detected protein content of 32.29 per cent and carbohydrate of 9.85 per cent in milky mushroom. Similar observation was also made by Sivaprakasam *et al.* (1986).

Similar findings on the nutrient content in various mushrooms were reported by several workers. Singh *et al.* (1999) reported the nutrient content of *Agaricus* as 90.10 per cent moisture, 43.75 per cent protein, 0.53 per cent crude fibre, and 4.59 per cent carbohydrate. Nutrient content of *Lentinus* was recorded with 89.80 per cent moisture, 10.20 per cent dry matter, 2.45 per cent protein, 1.2 per cent fibre and 5.15 per cent carbohydrate (Upadhyay and Rai, 1999). In *Volvariella,* the nutrient composition was with 95 per cent moisture content, 42 per cent protein, 11.28 per cent fibre and 7.82 per cent carbohydrate (Singh *et al.*, 1996). Sharma and Doshi (1996) reported nutrient content of a wild edible mushroom *Phellorinia inquinans* with 88.65 per cent moisture content, 11.35 per cent dry matter, 23.25 per cent protein, 12.66 per cent crude fibre and 9.00 per cent carbohydrate.

The total soluble solids of the native isolates ranged from five to seven which is in agreement with findings of Arumuganathan *et al.* (2003). They reported total soluble solids of 5 – 7 in *Agaricus bisporus.*

5.7 CULTURAL STUDIES

5.7.1 Evaluation of Growth of *Calocybe* sp. in Solid Media

The selected isolate, OE-349 was tested for their potentiality in supporting mycelial growth with different solid culture media *viz.*, potato dextrose agar, malt

extract agar, oat meal agar, coconut milk agar and jackfruit seed kernel agar. Fluffy nature of mycelium was observed in oatmeal agar followed by potato dextrose agar. Feable mycelial growth was observed in malt extract agar. These are in agreement with the findings of Suharban (1987) who has reported oat meal and potato dextrose agar as effective media for the growth of *Pleurotus* sp. Mehta and Bhandal (1992) reported better mycelial growth of *Auricularia* in PDA and wheat extract agar.

Radial growth of OE-349 on various media indicated PDA as the best medium followed by jackfruit seed kernel agar. The results are in agreement with the findings of Rafique *et al.* (1999) who concluded potato dextrose agar medium as the best for growth of different species of *Pleurotus i.e.*, 9 cm growth in 10 days time. Singh *et al.* (2000b) also observed potato dextrose agar as the best medium for radial growth of *Pleurotus sajor-caju* and *P. sapidus* which took only seven days for full growth in petridish. Sharma and Jandaik (1984) also reported better growth of *Pleurotus eryngii* on natural media like PDA.

Jackfruit seed kernel agar was equally effective as PDA in supporting mycelial growth of *Calocybe*. Pramod (2004) reported jackfruit seed kernel agar as the best media for *Volvariella volvacea* giving maximum growth of 7.66 cm in five days.

Oat meal though had supported fluffy growth of the culture had lower growth in malt extract agar. This result is contradictory to the observations made by Sharma *et al.* (2004), who reported malt extract agar as the best for the mycelial growth of *Agrocybe aegerita*. Similar observations were made for malt extract agar for supporting mycelial growth by Das *et al.* (2000b) and Yadav *et al.* (2003b) on *Agaricus bisporus*.

5.7.2 Growth of *Calocybe* in Shake and Static Culture

The study conducted to evaluate biomass produced in shake culture and static culture revealed that static culture produced comparatively higher biomass

than shake culture. This result was contradictory to the result obtained by Suharban (1987) who find out shake culture as a better method than static culture.

The jackfruit seed kernel broth produced higher biomass both in static and shake culture which is in accordance with the observations of Pramod (2004). The jackfruit seed kernel broth was followed by coconut milk broth which gave a biomass of 2.14 g. This result is in accordance with the findings of Ramos (1967) who reported the addition of coconut milk into the liquid media for enhancing the mycelial growth of *Volvariella volvaceae*. Similar observation on the enhancement of mycelial growth of *Pleurotus* has been reported by Balakrishnan (1994) and Anitha (1998). The potato dextrose broth and oat meal broth supported medium growth of *Calocybe*. The least biomass production was noticed in malt extract medium. Suharban (1987) also observed malt extract broth as unsuitable for mushroom mycelial production. But in contradictory to this result, Ishikawa (1967) reported malt extract medium as best for growth of *Volvariella*.

The reason for the higher biomass production in jackfruit seed kernel broth may be attributed to presence of sugars like fructose, dextrose which are preferred carbon sources and large amount of protein in jackfruit seed kernel.

5.7.3 Growth of Native Isolates of *Calocybe* in Different Carbon Sources

Of the seven different carbon sources tested for their efficacy in biomass production of *Calocybe*, fructose was the best source, followed by dextrose and mannitol. This result was in accordance with the finding of Chandra and Purkayastha (1977). They reported mannitol, glucose/dextrose and fructose supported excellent growth of *Agaricus campestris, Lentinus, Calocybe indica, Volvariella*. Fructose was more or less a good carbon source for all mushrooms. Fructose at two per cent gave higher mycelial biomass of *Calocybe*. The findings of Kaur and Lakhanpal (1995), Hong *et al.* (1981), Saha and Samajpati (1987), Khanna and Garcha (1985), Kumar and Mehta (1985) and Kumar and Munjal (1980) also confirmed glucose and fructose as best carbon sources for different mushrooms.

Inositol did not produce much mycelial dry weight, which is in accordance with the findings of Khanna and Garcha (1985) except mannitol, other sugar alcohols did not support growth of *Pleurotus*.

Lactose and sucrose was equally ineffective in supporting the mycelial growth of *Calocybe*. This is in accordance with the results obtained by Saha and Samajpati (1987) and Tan and Chang (1989). They observed lactose and sucrose as the least suitable carbon source for biomass production of *Tricholoma*, and *Lentinus*. The low mycelial biomass for lactose and sucrose may be attributed to the preference of monosaccharides over disaccharides by mushroom as they are easily degradable. Structural differences between sugars can be used to explain difference in the growth of native isolates of *Calocybe*.

5.7.4 Influence of Different Nitrogen Sources on the Growth of *Calocybe*

Among the organic and inorganic sources, organic sources were preferred by *Calocybe* for the production of mycelial biomass. Of these organic sources, peptone added media produced maximum mycelial biomass. This result is in accordance with the findings of peptone as best source of organic nitrogen for different mushroom sp namely *Pleurotus* (Khanna and Garcha, 1983), *Pleurotus ostreatus* (Mitra and Nandi, 1989), *Volvariella diplasia* (Banerjee *et al.*, 1990; Gupta *et al.*, 1996; Banerjee and Samajpati, 1989).

Among the inorganic sources, nitrates were preferred over ammoniacal forms. This is in accordance with the finding of Khanna and Garcha (1983). Different nitrate sources used were KNO_3 and $NaNO_3$ of which KNO_3 gave better mycelial dry weight than $NaNO_3$. This result is in agreement with finding that KNO_3 was a better source than $NaNO_3$ in *Pleurotus* (Jandaik and Kapoor, 1976), *Calocybe indica* (Chandra and Purkayastha, 1977), *Volvariella* (Ghosh and Sengupta, 1976; Mitra and Nandi, 1989) and *Agrocybe aegerita* (Sharma *et al.*, 2004).

Among the ammonium salts, nitrate, chlorides and carbonates were poor yielders of mycelial biomass of *Calocybe*. Bano and Srivastava (1970) reported

ammonium nitrate and chloride as poor N source for the growth of *P. flabellatus*. Similar observation was made by Jandaik and Kapoor (1976) on the growth of *Pleurotus sajor-caju*.

5.7. 5 Effect of H+ ion Concentration on Growth of *Calocybe*

Of the nine pH ranges tested both in acidic and alkaline range, slightly acidic pH of 5.5 gave maximum mycelial biomass of *Calocybe*. Increase of pH or decrease in pH resulted in the decrease of the mycelial biomass. This is in accordance with the results of experiment conducted by Chandra and Purkayastha (1977). They stated that pH 5.5 was optimum for the maximum mycelial biomass production of *Calocybe indica, Agaricus campestris, Lentinus, Volvariella* etc. They also found decrease in mycelial biomass with increase or decrease in pH.

Suharban (1987) and Rafique *et al.* (1999) proved that pH 5.5 was the best for the optimum mycelial production of all species of *Pleurotus*.

Similar observations were made on pH 5.5 as optimum for biomass production of *Volvariella* by Gupta *et al.* (1996). Mehta and Bhandal (1992) reported pH 6.0 for the optimum growth of mycelial biomass production in *Auricularia polytricha*.

5.8 IMPROVEMENT OF PRODUCTION TECHNOLOGY

5.8.1 Screening and Selection of Substrates based on Different Sterilization Procedure

Three different substrates paddy straw, spent mushroom substrate (SMS) and coir pith were subjected to different sterilization procedures *viz.*, boiling, solarisation and chemicals. The mycelial growth did not vary between the substrate. However solarisation of substrates especially SMS and paddy straw gave better mycelial growth and took minimum period for spawn run, initial production and harvest. Coir pith when subjected to boiling and solarisation resulted in the complete crop failure due to infestation by *Trichoderma*. But coirpith sterilized by carbendazim and formalin was found free from the infection by *Trichoderma*.

Solarisation proved to be a better sterilization technique for the substrates compared to boiling and chemicals. Spent mushroom substrate received back from the cultivation of oyster mushroom had lesser lignocellulolytic materials when compared to paddy straw.

5.8.1.2 Yield Characters

The isolate OE-349 produced maximum number of sporophore, higher yield and highest biological efficiency (BE) in SMS, followed by paddy straw. Cellulose, hemi cellulose and lignin are the major constituents on which the mushrooms grow. They have to depolymerise it by producing extra cellular enzymes. In this way the capability of a mushroom to grow on a particular type of substrate lies on its ability to degrade it which in turn is decided by the repertoires of the enzymes, the mushroom possesses. This decides the preference of a substrate over the others. Paddy straw is a rich source of lignocellulolytic materials with wide C : N ratio. *Calocybe* having only reduced capacity to produce cellulases to breakdown the cellulolytic materials. Once the paddy straw is used for cultivation of *Pleurotus*, the substrate gets degraded easily. *Calocybe* can absorb degraded nutrients from the spent mushroom substrate. These spent substrate obtained from *Pleurotus* cultivation had significantly higher content of major nutrients NPK than the base material used as substrate (Bhavana and Thomas, 2003). For easy assimilation of nutrients C: N ratio of the substrate should be low. In paddy straw it is 78.98 per cent. It was reported that during microbial utilization of plant substrates, nitrogen becomes mobilized into the cells of microbes and carbon released as carbondioxide. As a result of nitrogen being captive in organic combinations, the percentage of nitrogen continuously rises, hence C : N ratio reduces. This is in accordance with the findings of Kumar *et al.* (2000), who reported decrease in C : N ratio in paddy straw by *Pleurotus* cultivation.

This is in accordance with findings of Balakrishnan and Das (2001), who reported successful cultivation of milky mushroom in vallams using spent mushroom substrate.

Among the different sterilization techniques, solarisation gave maximum number of sporophores, yield and BE in *Calocybe*. It is an effective technique as the substrate will be free from chemical residue. Effectiveness of solarisation may be due to high temperature obtained for three days *i.e.*, effective pasteurization. This is in agreement with the findings of Ram and Thakur (2005) who concluded solarisation as method of sterilization for getting a BE of 69.43 per cent though it was lesser when compared to sterilization by chemicals and autoclaving. Solarised spent mushroom substrate followed by paddy straw solarisation gave higher yield and biological efficiency. This is in contradiction to the result obtained by Pandey and Tewari (2003). They observed maximum sporophore, number and yield of *Calocybe indica* in solarised paddy straw.

Chemical sterilization of substrates (paddy straw, SMS, coirpith) produced higher yield and biological efficiency when compared to boiling but was less effective than solarisation. Chemical sterilization did not produce desirable results which may be attributed to factors like temperature, treatment concentration and due to selective efficacy against undesirable fungi in mushroom cultivation. By continuous undesirable fungi develops resistance against carbendazim and formalin which also resulted in improper sterilization. Sharma *et al.* (1999) also reported adverse effect of chemical sterilization on the yield of mushrooms. Observations made by Gokulapalan *et al.* (1989), Singh *et al.* (1991) and Shukla and Biswas (2000) revealed chemical sterilization as a better method when compared to steam pasteurization in oyster mushroom cultivation. Similar observations were made by Yadav *et al.* (2003d) on the effectiveness of chemical sterilization followed by steam and solar sterilization in producing maximum yield and biological efficiency of *Tricholoma*.

Lowest yield and biological efficiency was noticed in boiling. The major reason for failure of boiling is attributed due to improper sterilization and also the excess moisture present in the substrate after cooking. This is contradictory to the observations made by Dubey (2000) who concluded steam pasteurization of paddy straw as the best for maximum sporophore production in *Pleurotus*.

Coir pith proved to be a unsuitable substrate for cultivation of *Calocybe,* due to heavy incidence of contaminants. The finding is in line with the result of Sherin *et al.* (2004) who stated composted coir pith was very poor in supporting milky mushroom production. They also reported heavy incidence of *Coprinus,* and *Trichoderma* in beds. Pramod *et al.* (2004) reported failure of coir pith as substrate to produce fruiting bodies of *Volvariella* sp.

Average fruiting body weight

It was observed that average fruiting body weight of *Calocybe* in solarised SMS and paddy straw was significantly higher than the chemical or boiling method. In contradictory to this results Pandey and Tewari (2003) and Yadav *et al.* (2003d) obtained higher fruiting body weight in steam sterilized straw than solarised straw.

5.8.2 Effect of Supplements on Yield of *Calocybe* sp.

Mushroom substrate is considered a self sufficient growing medium for the mushroom, but still it responds positively to externally added nutrient. Various organic supplements *viz.*, neem cake, rice bran, vermicompost @ 2 per cent and 4 per cent and ferrous sulphate at 5 ppm and 10 ppm were tested for their efficacy in supporting higher yields of *Calocybe*. The organic supplements except rice bran did not significantly affect the yield. Hazarika (1998) stated the organic additives had positive effect on crop yield of *Volvariella volvacea* and *Pleurotus sajor-caju.*

Among different supplements, rice bran was found superior to all the other supplements. Maximum number of sporophores was obtained when SMS was supplemented with 4 per cent rice bran. This is in accordance with the results of Balakrishnan and Das (2001). They reported 85 per cent BE in *Calocybe* when SMS substrate was supplemented with rice bran (20 per cent). Rao (1991) reported enhanced yield of *Pleurotus citrinopileatus* with 4 per cent rice bran as supplement. El-Kattan *et al.* (1991) reported 55.2 per cent biological efficiency of oyster mushroom by supplementation with 0.45 per cent rice bran.

Paddy straw supplemented with five per cent rice bran enhanced yield of *Volvariella* (Kaur *et al.*, 2004) and *Calocybe* (Eswaran and Susan, 2003). Similar findings were also observed by Gunasegaran and Graham (1987).

Supplementation of the substrate with vermicompost, neem cake and ferrous sulphate was found to be on par in respect to yield characters. In contrast to above result several workers reported neem cake as a better supplement than rice bran (Marimuthu *et al.*, 1994; Srivastava and Singh, 1999; Geetha *et al.*, 2002a).

Ferrous sulphate application resulted on the lowest biological efficiency of *Calocybe.* This result is not in agreement with the finding of Khanna *et al.* (2001) who recommended application of Fe^{+2} (5 ppm) at the time of casing to improve the yield of *Agaricus bisporus*. Sharma *et al.* (1994) reported increased biological efficiency of *Calocybe indica* by the addition of salts of $FeSO_4$ and $ZnSO_4$.

Application of supplements @ 4 per cent gave significantly higher yield than at 2 per cent rate. Geetha and Sivaprakasam (1994) stated that all organic amendments showed better effect at two or four per cent compared to higher concentration.

Incidence of weed moulds was noticed especially in beds supplemented with neemcake and vermicompost. Excess of nitrogen in neemcake and vermicompost favours the incidence of these moulds. Excessive heating of substrates by supplementation stimulates growth of competitor molds which may limit the rate of supplementation and their benefits (Carroll and Schisler, 1976). Neem cake and vermicompost was the base materials for the mass multiplication of *Trichoderma.*

5.8.3 Effect of Casing

Various casing materials *viz.*, coirpith soil mixture, vermicompost soil mixture, clay soil mixture and sand: soil: $CaCO_3$ mixtures were evaluated for their efficacy in supporting higher biological efficiency of *Calocybe*. Casing layer

plays an important role in inducing fructification by changing the vegetative phase to reproductive phase.

Maximum number of sporophore yield and biological efficiency was obtained in control (sand, soil, $CaCO_3$). It produced highest BE of 66.31 per cent. The growing mycelia produce metabolites in the substrate which decrease the pH. To maintain the pH of the substrates addition of $CaCO_3$ is necessary. This finding is in accordance with the reports of Purkayastha *et al.* (1981) and Purkayastha (1985). They recommended use of casing material with sand : soil in combination 12 per cent $CaCO_3$. Chakravarty *et al.* (1981) made similar observations in the use of casing material consisting of soil sand mixture whose pH was adjusted to 7.5 by the addition of $CaCO_3$.

Coirpith as casing material produced less number of sporophores with low biological efficiency. This is in agreement with findings of Suman and Paliyal (2004). They observed that coirpith when used as casing material in *Agaricus* cultivation yield was very low. Coirpith has the highest water holding capacity, and less porosity resulting in water logging, poor gaseous exchange and ultimately low yield. Coirpith was found deficient in macro and micronutrient hence could not support the growth of *Agaricus bisporus*.

Lowest biological efficiency was recorded in clay - soil mixture. The result is in accordance with the observation of Sharma *et al.* (1999) on the usage of clay soil as casing material in *Agaricus* cultivation. A preferable casing material is one which has minimum bulk density and particle density. Clay soil having high bulk density and particle density reduces free gaseous exchange which results in water logging. They observed that use of clay soil mixture as casing material in *Agaricus* cultivation resulted in quick absorption and very slow release of water by clay soil ending in water logged condition that hampered mushroom production remarkably. .

Coirpith and vermicompost when used as casing material had high contamination rate of *Trichoderma.* Since coir pith and vermicompost are uitlised as the base material for the mass multiplication of *Trichoderma*, it favours heavy

incidence of contaminants. Ramamoorthy *et al.* (1999) reported coir pith served as a carrier for the delivery of biocontrol agent namely *Trichoderma.* Coir pith and vermicompost, rich in organic matter, favoured the luxuriant growth of contaminants (Suman and Paliyal, 2004). .

5.9 STRAIN IMPROVEMENT

5.9.1 Irradiation

5.9.1.1 Irradiation with UV Rays

Non-irradiated control had fluffy mycelial growth when compared to irradiated ones. Irradiation with UV did not significantly affect the radial growth of culture but was slightly higher than the control. The irradiation of culture 5 cm away from source did not significantly increase the yield of *Calocybe.* But irradiation at 10 cm for 20 min produced maximum yield of *Calocybe* which was on par with control treatment. It has been proved that irradiation at 10 cm was effective in increasing the yield. Low yield obtained on other treatments were due to the low period of exposure. If more period of exposure *i.e.,* upto 90 minute if provided may result in more positive change than lower period of exposure. Stroller and Stauffer (1953) did not get desirable mutants of *Agaricus campestris*, when their spores were irradiated with ultra violet rays. Irradiation of cultures with UV at two distances for different periods enhanced proximate constituents like moisture content, dry matter, protein, total soluble solids, fibre content etc. Nambi *et al.* (2002) observed enhanced yield of *Calocybe indica* by UV irradiation for 45 minutes.

5.9.1.2 Irradiation with γ Rays

5.9.1.2.1 Mycelial Growth of Irradiated Culture of *Calocybe*

The culture irradiated at different KR levels were tested for the proliferation of mycelia in culture media and radial growth. It was found that the culture irradiated at 2.5 KR showed maximum mycelial proliferation. The radial mycelial growth of 5 - 7 cm was covered by the isolates in a week period. The result is in accordance with result obtained by Bahukhandi and Munjal (1988).

They noted that mycelial growth of irradiated cultures were slow growing and fine appressed growth, whereas in non- irradiated control it was fast growing hypae. Ravichandran and Muthusamy (2005) reported that irradiated culture took longer time to complete growth of *P. florida* when compared to control.

Kalra and Phutela (1991) reported that only fast growing strandy cultures could be the best isolates of *Volvariella.* This result obtained is in contradiction with findings of Pramod (2004), who reported maximum mycelial growth in 2.5 KR irradiated *Volvariella* cultures.

5.9.1.2.2 Yield Performance

The yield performance of irradiated isolates at different levels was compared with the control. It was obvious from the result that there was an increase in yield with increase in dosage whereas at higher dosages, number of sporophores, yield and BE of *Calocybe* was reduced. This is in accordance with findings of Sarala (2001) who reported increased yield of *Pleurotus* up to 1KR and decline was observed with increased dosage. Pramod (2004) reported reduction in yield of *Volvariella* with increase in dosage from 0.5 – 2.5 KR. Ravichandran and Muthusamy (2005) reported irradiation dosage of 2 KR gave maximum yield, of *Pleurotus florida* but increased dosage resulted in reduced yield.

Bahukhandi and Munjal (1988) observed early fruit body initiation of *Pleurotus sajor-caju* on irradiation with gamma rays for 5 minutes.

The result of study indicated that the increased dosage of irradiation manipulated the genetic make up of the culture, which may have resulted in reduced yield of mushroom. *Calocybe* could tolerate only upto 2 KR for producing maximum yield.

5.9.1.2.3 Proximate Constituent

Irradiation of *Calocybe* isolate at different level from 0.5 KR – 2.5 KR resulted in the reduction of moisture content, whereas dry weight of the cultures increased with increased dosage. Protein content of irradiated isolate was higher

than non irradiated, but there was an increase followed by a decline with increasing dosage. Fibre content also increased with increasing levels of irradiation. By irradiation there can be changes both in phenotype and genotype. Genotypic variations may result in change in protein types.

5.9.1.2.4 Keeping Quality

The keeping quality or shelf life of *Calocybe* was comparatively high when compared to other mushrooms due to its high fibre content. The shelf life can be further enhanced by irradiation with γ rays. Irradiation at 2 KR had enhanced the keeping quality of mushroom upto 37.33 when compared to control with 21.67days. The reason for enhanced shelf life of γ irradiated mushrooms was due to the increase in the fibre content and also reduced moisture content. The fibre content increased as the irradiation dosage increased but the moisture content had a reverse effect.

Roy and Bahl (1984) and Wahid and Kovacs (1980) observed increased shelf life of *Agaricus bisporus* as a result of gamma irradiation. Beelman (1988) opinioned about enhancing the shelf life of *Agaricus bisporus* from three to six days by gamma irradiation at FDA approved dose of 100 KR. Menniti (1976) also observed γ irradiation at higher dosages of 600 KR to be effective in prolonging the shelf life of *Agaricus campestris*.

5.9.2 Mating/Crossing of Isolates of Dissimilar Characters

5.9.2.1 Multispore Crossing

Hybridization of multispores / tissue was absent since there was no thick strands of mycelium at joining region. Verma *et al.* (2000) reported that raising of multi spore isolates may yield same variability due to the anastamosis of hyphae originating from different spore is not widely used for breeding purpose but to rejuvenate old cultures.

Bhandal and Mehta (1989) reported that growth was invariably slower in tissue culture and they tend to yield lesser than parent culture.

5.9.2.2 Single Spore

Isolation of single spores were done as described earlier. Bhandal and Mehta (1989) developed four single spore isolates of *Agaricus bisporus*. Similarly, Kalra and Phutela (1991) could raise and evaluate several single spore isolates of *Volvariella volvacea* and *V. diplasia*. Yadav *et al.* (2002) isolated four single spore cultures of *Agaricus bisporus* for development of genetically improved strains.

5.9.2.3 Hybridization

Of the several crosses A_1 x B_1, A_1 x B_2, A_2 x B_1, A_2 x B_2, A_3 x B_1 and A_3 x B_2 attempted, some of the crosses were found to be incompatible in nature. One of the persistent crosses, A_3 x B_2 was evaluated and yield parameters were compared. Yield character studies indicated higher number of sporophores, yield, and BE in hybrids than the parents. Similarly, nutrient status or proximate constituents were better in hybrids when compared to the parents. The enhancement in yield and nutrient status may be due to genetic change of hybrids by crossing. The results were in agreement with the findings of Salmones and Duran (2001). They reported crossing of monospores of *Pleurotus pulmonarius* to produce dikaryons with yield of 8.4 – 25.4 per cent in parents and 19 – 30.6 per cent in the progenies. Thakur and Bhandal (1992) reported that intermating of isolates of *Agaricus* crosses yielded better than the parents. The results of the nutrient content or proximate constituents were in agreement with findings of Periyasamy and Natarajan (2002). They reported crossing of single basidospore of *Pleurotus djamor* var *roseus* to produce dikaryons which showed better biological efficiencies, enriched proteins and minerals than the parental strains.

5.9.4 Enzyme Activity

Activities of peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase were estimated. The activity of peroxidase in hybrid was more or less similar to the parents. The activity of polyphenol oxidase in parents as well as hybrid was very low, unable to be detected in a native gel. Anandh and

Prakasam (2002b) noted that polyphenol oxidase activity attained peak during mycelial growth and decreased during sporophore production of *Calocybe indica* indicating the involvement with substrate degradation. The activity of polyphenol oxidase was maximum in substrate rich in lignin like sawdust, and coirpith compost (Krishnamoorthy *et al.*, 2002b and Krishnamoorthy *et al.*, 2005b). The activity of PAL was also very less. Since these are defense related enzymes, produced under stress condition, where in normal mushroom the activity of these are lacking. If there was stress condition there may be production of enzymes. *Calocybe* was the least effective fungus in the degradation of lignin (Ramamoorthy *et al.*, 1999).

5.10 MOLECULAR CHARACTERIZATION

5.10.1 Molecular Characterization Using Isozyme Analysis

Isozyme analysis for determining the genetic variation between parents and hybrid did not yield any result as the activity of these enzymes were less. The enzyme activity of mushrooms varied depending upon the substrate rather than between the isolates. This is in contrast to result obtained by Packia *et al.* (2000) who determined genetic variability of different strains of *Pleurotus citrinopileatus* based on isozyme electrophoresis.

5.10.2 Molecular Characterization Using RAPD Analysis

The DNA samples isolated from parents and hybrids were of good quality as analysed by agarose gel electrophoresis. The purity of DNA samples (OD_{260} to OD_{280}) indicated that the quality of DNA samples were sufficient for RAPD analysis.

Out of the eight RAPD primers, only four primers yielded products on PCR reaction. All the four RAPD primers were reported earlier by Singh *et al.* (2003b) in *Calocybe*. But only two primers (OPA-04 and OPP-12) gave reproducible results. These two primers could very well distinguish the hybrid from both the parents. The two parents were also differentiated by these two primers. The primer OPA-04 yielded a monomorphic band in the hybrid and

parent B_2. Similarly OPP-12 yielded three monomorphic bands in the hybrid and parent B_2 and these results indicated the hybrid is more close to the parent B_2.

The non-reproducibility of PCR amplification of the DNA samples from the parent and hybrids with the other reported primers may be due to the variations in the reaction conditions provided by the thermal cycler or the quality of the DNA samples and chemicals used.

The statistical analysis of the RAPD data also revealed the genetic relatedness and the distance between the parents and hybrids. As the dendrogram indicated the hybrid and one of the parents grouped into a single cluster and the other parents formed a separate cluster. This also indicated that the hybrid is more similar to the parent B_2.

So these two RAPD primers (OPA-04 and OPP-12) can be used for distinguishing *Calocybe* parents and the hybrid generated in the future studies also.

5.11 PEST

Snail was reported as a major pest of milky mushroom as it seriously affected the sporocarps. Those mushrooms which were attacked by snails had stunted growth, discolouration, and the stipe became hollow. Snails could be effectively managed by spraying 0.5 per cent salt solution or by putting 5 g salt on the snail if they are seen on the beds. Snail was reported as a serious menace in *Calocybe* cultivation by Heera *et al.* (2006).

Summary

6. SUMMARY

Surveys were conducted during pre-monsoon and post monsoon periods in the districts of Thiruvananthapuram, Kollam and Pathanamthitta for the collection of native strains of *Calocybe* during the year 2003-2004. Seven isolates were obtained from Thiruvananthapuram, two from Kollam and one from Pathanamthitta, *viz.*, OE-349, OE-350, OE-351, OE-352, OE-353, OE-354, OE-355, OE-356, OE-357 and OE-358. The collected samples were brought to the laboratory and subjected to various morphological observations including macroscopic and microscopic characters. The tissue isolation was done for obtaining the pure culture of ten isolates of *Calocybe*.

Mycelial growth of native isolates of *Calocybe* indicated that isolates OE-349, OE-350, OE-355 and OE-357 had better growth on petridish and PDA slants. These isolates completed full growth in petridish in a fortnight period, compared to other isolates which took longer periods.

Nature of mycelial growth on grains was better with the above mentioned isolates and feable growth on the isolates OE-352 and OE-354.

The time taken for full growth of mother spawn varied widely among different isolates. The isolate OE-355 took minimum period, whereas OE-353 maximum period (26.67 days).

Characters of the isolates on beds were assessed using different observations. Days taken for initial appearance on cased bed varied from 8.33 – 20. The maximum number of days was taken for isolate OE-356 and minimum period for OE-349. The days taken for harvest of the native isolates were maximum in the isolate OE-349 and maximum in the isolate OE-356. The days taken for harvest from bed preparation was minimum for the isolate OE-349 and maximum for OE-356.

The native isolates *viz.*, OE-349 - OE-358 were tested for their fruit body characters and yield parameters and found that native isolates varied in their performance. The native isolates namely, OE-349, OE-357 and OE-350 were highly suited for cultivation under Kerala conditions. These out yielded all the other isolates. The isolate OE-355 and OE-354 had very low yield.

Maximum numbers of sporophores was obtained in the isolate OE-349 which had lower mortality percentage when compared to other isolates and biological efficiency.

Only single sporophore was obtained from isolate OE-355 which had lowest biological efficiency. The mortality percentage of native isolates varied from 55.55 to 90.85 percentage.

The characters of the native isolates were studied by raising bed. The isolates showed wide variation in stipe length, stipe diameter pileus length and thickness, gill/cm and average fruit body weight. Among the ten isolates OE-349 was the best.

The moisture content of native isolates ranged between 80.92 and 89.64 per cent with maximum in isolate OE-351 and minimum with the isolate OE-355. The dry matter content showed the reverse trend of moisture content with maximum in the isolate OE-355 and minimum in the isolate OE-351. The total soluble solids content though did not vary significantly among different isolates. The lowest fibre content was observed in the isolate OE-349 (16.02 per cent) and highest in OE-356 (24.1 per cent). The protein content in native isolates of *Calocybe* estimated by Bradford method indicated that isolate OE-349 had the maximum amount and lowest in the isolate OE-356. The lowest carbohydrate content was expressed in isolate OE-356 and highest content on OE-354.

The keeping quality of native isolates varied widely. It could be stored in perforated polythene covers in refrigerator for three to four weeks.

Five different solid culture media were tested for their potentiality in supporting the radial mycelial growth of *Calocybe*. Among these media, oat meal agar gave the maximum fluffy growth. Potato dextrose agar and jackfruit seed kernel agar were found to be very effective in supporting the mycelial growth. Malt extract agar was ineffective in supporting the mycelial growth.

Biomass production of *Calocybe* on liquid broth revealed that static culture was better when compared to shake culture. Jackfruit seed kernel broth was superior in both static and shake culture in maximum biomass production and least biomass on malt extract broth.

Studies on the effect of carbon sources on biomass production of *Calocybe* revealed that fructose was the best source followed by dextrose. The isolate could not effectively utilize sucrose and lactose. Organic nitrogen sources like peptone and beef extract were preferred by *Calocybe* over inorganic sources. Among the inorganic sources, nitrate sources were preferred to the ammoniacal ones. Studies on the H^+ ion concentration (pH) showed that *Calocybe* prefers an acidic pH of 5.5 – 6.0 for maximum biomass production than the alkaline ranges.

Three different locally available agrowastes *viz.*, paddy straw, spent mushroom substrate and coir pith were subjected to different sterilization techniques *viz.*, boiling, solarisation and chemical. Paddy straw and spent mushroom substrate sterilised by solarisation took minimum period for spawn run, initial appearance and harvest. Solarised spent mushroom substrate gave maximum number of sporophores, yield, biological efficiency and low mortality percentage of the initials followed by solarised paddy straw. Least suitable substrate for the cultivation was found to be coir pith, subjected to boiling and solarisation as it did not produce any sporophores, because of high incidence of *Trichoderma*.

Effect of supplements on the yield of *Calocybe* were tested with neem cake, rice bran, ferrous sulphate and vermicompost at different rates. It was

observed that rice bran @ four per cent gave maximum number of sporophores, yield, biological efficiency and inhibited the contaminant growth which was followed by two per cent rice bran. The supplements neem cake and vermicompost favoured the incidence of competitor mould *Trichoderma*.

Casing is essential for the proper fructification of *Calocybe*. Studies conducted in the effect of casing on the yield characters of *Calocybe* reveals that sand, soil, $CaCO_3$ mixture used as casing material gave maximum number of sporophores, yield and biological efficiency. Casing material consisting of clay - soil mixture did not yield any sporophores, while the use of coir pith and vermicompost used as a constituent in casing material induced heavy *Trichoderma* contamination.

Strain improvement was attempted with irradiation using UV rays and γ rays. Irradiation of cultures with UV rays reduced the fluffy nature of mycelial growth. Irradiation of *Calocybe* culture with UV rays had a depressing effect on the yield parameters like number of sporophores harvested, yield, and biological efficiency (BE) when compared to the non-irradiated culture. Irradiation at 10 cm away from the source for 20 minutes gave comparatively higher yield but was lower than the control. The time taken for mushroom production was enhanced by irradiation with UV rays. Irradiation with UV had a significant effect on the nutrient status. There was a decline in the moisture content and increase in the dry matter content as a result of irradiation. Enhanced amount of protein content was observed in irradiated culture when compared to control. The total soluble solids did not vary between irradiated and non-irradiated cultures. The fibre content of the irradiated cultures showed an increasing trend with an increase in the exposure period. The keeping quality of the mushroom at room temperature and refrigerator did not show variation between irradiated and non-irradiated culture.

Irradiation with γ rays showed better fluffy nature of mycelial growth in an irradiated culture than in non-irradiated cultures. Increase in irradiation dosage enhanced growth of culture in petridish.

Irradiated cultures showed increased time for mushroom production. Yield parameters like number of sporophores, yield and biological efficiency were enhanced with irradiation dosage but a decline was observed after a certain dosage. Maximum number of sporophores, yield and biological efficiency were observed in culture irradiated at 1.5 KR followed by 2.0 KR and 1.0 KR. Higher doses and lower doses reduced the yield when compared to control.

The nutrient status of mushrooms irradiated with γ rays also varied widely. The amount of moisture was reduced as a result of increase in irradiation dosage. The irradiation increased the dry matter content as a result of increased dosage. The protein content and fibre content were enhanced as a result of irradiation.

Studies on the shelf life of irradiated cultures showed enhanced keeping quality both at room temperature and refrigerator than the non-irradiated ones.

Crossing of isolates attempted with multispores resulted in complete divergence indicating incompatibility. Single spores isolates from different cultures hybridised using dual plate culture, resulted in the development of hybrids. The yield characters indicated that the hybrid produced more number of sporophores, yield and biological efficiency when compared to parents.

The nutrient composition studies showed that hybrid contained less moisture, and more dry weight than that of the parents. The hybrid also showed enhanced amount of protein when compared to both the parents. The total soluble solids was not high on hybrid when compared with its parent. The fibre content of hybrid was lower than that of both the parents.

The enzyme activity (PAL, PO, and PPO) of hybrids was not significantly different from that of the parents.

Molecular characterization using isozyme analysis did not yield any result since the bands in gel were absent.

Molecular characterization using RAPD analysis, with eight primers indicated that the hybrid had 25.44 per cent similarity with parent A_3 and 44.4 per cent with parent B_2. This was derived using a dendrogram which grouped the hybrid and one of the parents into a single cluster and the other parents formed a separate cluster. This also indicated that the hybrid is more similar to the parent B_2.

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Appendices

APPENDIX – I

DATA-SHEET

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/Cylindrical/Solid/Hollow/Slender

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

Score card

Appearance	
Excellent	5
Good	4
Fair	3
Poor	2
Very poor	1
Colour	
Very good	5
Good	4
Fair	3
Poor	2
Very poor	1
Texture	
Very soft	5
Soft	4
Slightly fibrous	3
Fibrous	2
Very fibrous	1
Flavour	
Highly acceptable	5
More acceptable	4
Acceptable to certain extent	3
Less acceptable	2
Not acceptable	1

APPENDIX – III

Composition of different media

a) Potato dextrose agar (PDA)

Potato	:	200 g
Dextrose	:	20 g
Agar-agar	:	20 g
Distilled water	:	1 l

b) Malt extract agar

Malt extract	:	25 g
Agar-agar	:	20 g
Distilled water	:	1 l

c) Oat meal agar

Oats	:	40 g
Agar-agar	:	20 g
Distilled water	:	1 l

d) Jackfruit seed kernel agar

Jackfruit seed kernel	:	200 g
Dextrose	:	20 g
Agar-agar	:	20 g
Distilled water	:	1 l

e) Coconut milk agar

Coconut milk	:	400 ml
Agar-agar	:	20 g
Distilled water	:	1 l

STRAIN IMPROVEMENT AND PRODUCTION TECHNOLOGY OF MILKY MUSHROOM (*Calocybe indica* P. &C.)

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ABSTRACT

Survey conducted in different localities to obtain the native flora of *Calocybe* resulted in the collection of ten isolates of *Calocybe,* seven from Thiruvananthapuram, two from Kollam and one from Pathanamthitta.

The nature of growth in petridish and spawn substrate of the native isolates varied widely. Of the ten isolates, the most promising isolate OE-349 which gave better yield, shelf life, nutrient status and low mortality was selected for further studies.

Cultural characters studied showed that locally available jackfruit seed kernel broth can be substituted for potato dextrose broth for maximum biomass production. In solid media the *Calocybe* isolate had fluffy growth in oat meal agar and better radial growth in potato dextrose agar medium.

Calocybe preferred fructose, rather than dextrose, hence can be used for higher biomass production. Least preferred carbon sources were lactose and sucrose. Among the nitrogen sources peptone was the best for maximum biomass production. The inorganic source, ammonium salts were less preferred than nitrate source. *Calocybe* preferred a pH of 5.5 for maximum biomass production. An increase or decrease in pH resulted in the reduction of biomass.

Among the various substrates subjected to different sterilisation techniques, spent mushroom substrate (SMS) subjected to solarisation out yielded paddy straw and coir pith, compared to boiling and chemical methods. Solarised SMS gave higher biological efficiency and better fruiting body weight. Mortality percentage was minimum in solarised SMS. Solarised beds of SMS and paddy straw were free from contaminants. Coirpith did not prove as a successful substrate for *Calocybe* cultivation as it was highly contaminated with *Trichoderma.*

Of the various supplements, rice bran supported higher biological efficiency than others. The application of supplements at four per cent rate gave

better yield than two per cent. Rice bran supplementation of solarised SMS out yielded the other supplements neem cake, vermicompost and ferrous sulphate. Neem cake and vermicompost were not suitable for supplementation as there was heavy incidence of *Trichoderma* on beds.

Casing materials consisting of sand, soil and $CaCO_3$ gave better biological efficiency than coir pith soil mixture, vermicompost-soil mixture and clay-soil mixture. Vermicompost and coir pith when used as an ingredient in casing material there was heavy incidence of *Trichoderma*. Clay-soil mixture did not support fruiting body production of *Calocybe* due to crust formation as a result of quick absorption and slow release of water.

Strain improvement was done using UV rays and gamma irradiation. UV irradiation at two distances (5 cm to 10 cm) from source for different periods (10, 15, 20 and 25 minutes) did not produce any significant effect on the yield as well as shelf life and nutrient status of mushroom. But irradiation at 10 cm from source had a positive effect on nature, radial growth of mycelium and yield when compared to irradiation at 5.00 cm.

Irradiation with γ rays enhanced yield, nutrient status and shelf life of *Calocybe*. Irradiation with γ ray from 0.5 – 2.5 KR showed an increase in yield upto 1.5 KR followed by a decline.

A hybrid was developed by crossing single spores of two different isolates with dissimilar character. The hybrid (54.88 per cent BE) out yielded the parents in yield, characters, nutrient content and enzyme activity.

Molecular characterisation of hybrid and their parents revealed that hybrid had 44.40 per cent similarity with one parent and 28.57 per cent similarity with other parent. Dendrogram constructed clustered one parent and hybrid into one group and other parent into another group at 0.26 similarity coefficient.