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Flammulina velutipes, the culinary medicinal winter mushroom

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FOREWORD

The land resources in the world for raising food crops are limited and there is no possibility for its further increase. This warrants a wise use of land with due regards to its sustainability for long term productivity. Keeping in view this limitation mushroom cultivation is a wonderful activity which fits very well in the present scenario and also alleviates environmental pollution besides producing a highly valuable protein rich product. India is blessed with varied agro climate, abundance of agricultural waste and manpower making it the most suitable for the cultivation of all types of mushrooms. Our Centre is giving greater emphasis on the diversification in mushroom cultivation. Farmers are being motivated to cultivate these new mushrooms for better returns. Diversification will provide opportunity to the seasonal mushroom growers for round the year cultivation and utilization of different cultivation substrates. I appreciate the efforts and labour put in by the authors in compiling and editing the Bulletin for its use by the mushroom growers and researchers.

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Dated:

INTRODUCTION

Varied agroclimatic conditions and availability of agricultural and industrial wastes in India offer great opportunities for cultivating different mushrooms on commercial scale. Introduction of new mushrooms is also important to meet out the increasing public appetite for new and different foods. Total world production of different mushrooms especially *Lentinula, Pleurotus*, *Auricularia* and *Flammulina* has gone up many folds in the last few years. Occasionally the glut of a particular commodity during bumper production period also fetches poor market price to the growers which happened in the early 1990 and made several growers search for alternative crops to supplement their income. Different mushrooms while adding variety, flavour and eye appeal to our food, mushrooms are also considered nutritious because of their proteins, fiber, vitamin and mineral content. A wide variation in the chemical composition of a particular mushroom has been observed by various workers which may be due to the environmental conditions, stage of sampling and method of analysis. Majority of the Indian population being vegetarian, consumption of mushrooms would certainly augument their diet which is deficient in proteins and minerals. Mushroom consumption can thus prove a boon to the growing children as well as breast feeding mothers.Some mushrooms have special significance for medicinal purposes. Specialty mushrooms have been traditionally used in China and Japan for medicinal and tonic purposes since time immemorial.Mushroom consumption has proved beneficial for the patients suffering from hypertension, high sugar and heart problems. Beneficial effects of shiitake consumption are known as enhanced vigour, energy, potency and diminished ageing. Therefore, the cultivation and consumption of mushrooms can contribute towards sound health.

Species of *Flammulina* have been reported to occur naturally on various deciduous tree species namely, poplar (*Populus* spp), willows (*Salix* spp), elms (*Ulmus* spp) plum (*Prunus* spp), maple (*Acer* spp) and birch (*Betula* spp) as a parasite and later as a saprophyte growing on the trunks or stumps of these broad-leaved trees from the end of autumn to early spring. Infection occurs only on wounded or weakened trees. Fruit

bodies appear after a few months at temps. between -2 and 14^{o}C and at under low intensity winter light (Poppe, 1974, Zadrazil1999).Under *in vitro* conditions fruit bodies were produced by culturing on potato dextrose agar in darkness at 30^{o} C for 7 days and subsequent transfer to 5^{o}C. (Chandra and Purkayastha,1972). *Flammulina velutipes* was cultivated as early as 800AD in China and today it is being cultivated all over the world especially in China, Siberia, Asia Minor, Europe, Africa, North America, Australia, Taiwan and Japan. This mushroom is particularly known for its taste and preventive as well as curative properties for liver diseases and gastroenteric ulcers. In addition, winter mushroom has also been reported to contain immunodomodulatory, antitumor and antibiotic substances

Common names: Enokitake, Enoki, golden needle mushroom, winter mushrooms, velvet foot, or velvet stem.

Taxonomic Position:

Kingdom: Fungi;Division: Basidiomycota;Class:Homobasidiomycetes;Order: Agaricales; Family: **Tricholomataceae** Marasmiaceae;Genus:*Flammulina;*Species**:** *callistosporioides, elastica, fennae, ferrugineolutea, mediterranea, mexicana, ononidis, populicola, rossica, similes, stratosa, velutipes*

Morphological and Molecular characterization:

Flammulina velutipes is long and thin white mushroom used in the Cuisine of Japan and China. The mushroom naturally grows on the stumps of the Chinese hackberry tree, called *enoki* in Japanese, but also on some other trees as for example mulberry and persimmon trees. There is a significant difference in appearance between the wild and the cultivated mushrooms. Cultivated mushrooms are not exposed to light resulting in a white color, whereas wild mushrooms usually have a dark brown color. The cultivated mushrooms are also grown to produce long thin stems, whereas wild mushrooms produce a much shorter and thicker stem. Its fruit bodies are small but delicious. The pileus is 2-3 cm in diameter and hemispheric or convex in early stages of its development, gradually opening to a plane as it grows. The surface of the pileus is viscid when moist and is

yellowish brown or dark brown, usually with a light brown margin. The flesh is almost white. The gills are white or light-cream colored, and they are adnexed or decurrent to the stipe. The stipe is stiff, 2-9 cm long and 2-8 mm in diameter. The lower part of the stipe is dark brown, while the upper part is light brown and sometimes almost white. The spores are white, with a flat surface, cylinderically oval, and 5-7 X 3-4 µm in size.

Neustroeva (1984) studied the morphology of 30 strains of *Collybia velutipes* collected from 11 tree host species. The strains fell into 2 distinct groups: the 1st group, consisting of isolates collected from linden (*Tilia cordata*) and elder (*Sambucus nigra*]) was characterized by more rapid growth. Three strains selected from each group produced fruit bodies within 23-25 days (group 1) and 40-50 days (group 2) after inoculation on a solid substrate.

Molecular characterization (Kong *et al*.,1997), revealed that various isolates of *F. velutipes* collected worldwide have two differently sized IGR (intergenic region) of 1.61 and 1.58 kb. Based on restriction analysis of the IGR and ITS(internal transcribed spacer) rDNA using 6 different 4-bp-recognizing enzymes, 22 isolates were classified into 4 groups with 92% similarity. In random amplification polymorphic DNA (RAPD) analysis with 20 primers, 68 collected isolates were classified into 7 genotypic classes with 87% similarity. Interestingly, 30 isolates producing white fruit bodies were classified into one group. Nucleotide sequence and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the ribosomal RNA gene (rDNA) regions containing the internal transcribed spacers (ITSs) and the 5.8S rRNA coding sequence was used to differentiate between 7 typical *Flammulina* strains (MH09201, MH09208, MH09210, MH09234, MH09286, MH09289 and MH09297). These nucleotide sequences revealed the presence of strain-specific deletions, insertions and substitutions (Palapala *et al*.,2002). RFLP patterns produced using restriction endonucleases DraI, FokI, HaeII, MboII, and NlaIV, enabled identification of specific *Flammulina* strains. PCR-RFLP analysis of the ITS regions appears to be a useful tool for the identification of *Flammulina* strains.Digestion of *F. velutipes* collections with two restriction enzymes, Bgl I and Bst UI, gave variable restriction fragment patterns and revealed biogeographically identifiable patterns. Three haplotypes of *F. velutipes* were identified

with geographical distributions chiefly in Asia, Europe and North America. (Methven *et al.,* 2000). Azuma et al.,1996. A cDNA library was constructed using mRNA from 7-day (7d)-old cultures and also screened for genes that may be involved in fruiting body differentiation. One cDNA clone, FDS (*F. velutipes* differentiation specific), was isolated by differential screening.

History of cultivation:

It is reported that (Yang,1986: Wang,1995), *F.velutipes* first cultivated in China during the 8th century. In 1928, Moriki cultivated it with sawdust and rice bran in Japan (Nakamura, 1981). The mushroom has been cultivated in Japan for over 300 years, initially on wood, and later in the bottles During the 1960s, its cultivation revolutionized in Japan, which became its largest producer in the world and enjoyed this position till the 1980s. Since the early 90's, China has occupied the first place in its production. It was estimated that in the Mainland China its production was about 200,000 tons during 1995 (Meiging, 1997). Production data from different other countries too indicated a faster growth rate in terms of its total production. In the United States, for example, the production of *Flammulina* increased at an estimated rate of 25% or more per year for the last four years (Royse, 1995). Production of *Flammulina* is based on synthetic substrate contained in polypropylene bottles or bags. The substrates most utilised are agricultural residues, such as corncobs, cottonseed husk, sugarcane bagasse, etc., besides sawdust (Chang, 1989; Yang, 1986; Fan *et al*, 1990; Wang, 1995; Royse, 1995).

World Production

Diversification of the mushroom industry, in terms of number and quantity of species cultivated, has accelerated worldwide during the 1980s and 1990s. Twenty years ago, 70% of the world's mushroom supply was *Agaricus bisporus*, but by the mid-1990s, this had fallen to only 37%. (Royse, 1997). Total mushroom production worldwide has increased more than 18-fold in the last 32 years, from about 350,000 metric tons in 1965 to about 6,160,800 metric tons in 1997 (Table 1). The bulk of this increase has occurred during the last 15 years. A considerable shift has occurred in the composite of genera that

constitute the mushroom supply.During the 1979 production year, the button mushroom, *Agaricus bisporus*, accounted for over 70 per cent of the world's supply. By 1997, only 32 per cent of world production was *A. bisporus*. The People's Republic of China is the major producer of edible mushrooms, producing about 14 million tons in 2006.

Table 1. World production of cultivated edible mushrooms in 1986 and 1997.

Fresh weight (x 1,000 t)		**Increase**	
Species	**1986**	**1997**	**(%)**
Agaricus bisporus	1,227 (56.2%)	1,956 (31.8%)	59.4
Lentinula edodes	314 (14.4%)	1,564 (25.4%)	398.1
Pleurotus spp.	169 (7.7%)	876 (14.2%)	418.3
Auricularia spp.	119 (5.5%)	485 (7.9%)	307.6
Volvariella volvacea	178 (8.2%)	181 (3.0%)	1.7
Flammulina velutipes	100 (4.6%)	285 (4.6%)	130.0
Tremella fuciformis	40 (1.8%)	130 (2.1%)	225.0
Hypsizygus marmoreus	— —	74 (1.2%)	—
Pholiota nameko	25 (1.1%)	56 (0.9%)	124.0
Grifola frondosa	— —	33 (0.5%)	—
Others	10 (0.5%)	518 (8.4%)	5,080.0
Total	**2,182 (100.0%)**	**6,158 (100.0%)**	**182.2**

Source: Chang (1999)

Table 1. World production of cultivated edible mushrooms in 1978 to 2002.

Year	**Fresh weight (x 1,000 t)**
1978	**10,600**
1983	**14,510**
1986	**21,760**
1990	**37,630**
1994	**49,093**
1997	**61,684**
2002	**122,500**

Source: Chang (2002)

Flammulina ranks at fourth place in the category of edible mushrooms for production and consumption. During 1990, its production was estimated to be approximately 143,000 tons, which increased to 230,000 tons in 1994, showing a remarkable jump of 61% (Chang 1996).Worldwide 1986 production of *F. velutipes* (enokitake) has increased from about 100,000 tonnes in 1986 to about 187,000 t in 1991 (87% increase).

Worldwide production of *F. velutipes* (enokitake has increased from about 143,000 metric tons in 1990 to about 285,000t in 1997 (a 2-fold increase, Chang 1999). Japan is the main producer of winter mushroom (Furukawa 1987). In 1986, Japan produced 74,387tonnes; by 1991, production had risen to 95,123 tones and, by 1997, Japan produced 174,100 tones -an increase of about 45% in six years. From these data, it is evident that other countries are enjoying a faster growth rate, in terms of total production. In the United States, for example, winter mushroom production has increased at an estimated rate of 25% or more per year for the last four years.

Lee and Park (1994) investigated the profitability of bottle mushroom cultivation in the Korea Republic and suggested that for a production level of 2000 bottles per day, it requires a total expenditure of worth 5 million and obtains an annual net income of worth 110 million. For a production level of 1000 bottles per day, *Pleurotus ostreatus* requires a total expenditure of worth 300 million and provides an annual net income of worth 30 million.

Nutritional value:

Flammulina velutipes is a popular edible mushroom in Japan where a tender cultivated form of the wild version is called "enokitake." It is commonly used in Japanese cooking and increasingly can be found in salads in gourmet restaurants. *F. velutipes* contains 31.2 per cent protein, 5.8 per cent fat, 3.3 per cent fiber and 7.6 per cent ash on the basis dry material.

Medicinal attributes and their properties

Flammulina velutipes, a delicious mushroom is rich in peroxidase, superoxide dismutase, and others and can prevent some severe diseases like cancer and coronary heart disease. It is beneficial for physical and intelligence of children. Winter mushroom contains compounds that prevent as well as cure liver disease and gastroenteric ulcers provided it

is taken on a regular basis (Ying *et al.*, 1987; Yoshioka *et al*., 1973). In addition, like many other specialty mushrooms, winter mushroom contains immunodomodulatory, antitumor, tumor inhibiting, and anti-biotic substances. Both mycelium and fruitbody of *F. velutipes* could be recommended for formulating antioxidative dietary supplements.

Winter mushroom contains several types of amino acids including valine, which inhibits the growth of Ehrlich ascities tumour and sarcoma 180 in mice; and lysine. It also reported to increase body height and weight (Ying *et al.,* 1987). Winter mushroom contains a cardiotoxic protein (flammutoxin) that is rendered harmless when subjected to heat (100 degrees C) for 20 minutes. The dose is 8-9 g/day.

Purification of lysine and flammutoxin and revealed that it is a single polypeptide chain of Mr 32 000 and pK about 5.4 (Bernheimer and Oppenheim,1987). It contains large amounts of tryptophan, serine and glycine, and few or none of the sulphur-containing amino acids. The toxin appears not to be a phospholipase and it was not inhibitable by any of a variety of lipids. Flammutoxin caused efflux of potassium ions from human erythrocytes and swelling of the cells before haemolysis. Flammutoxin did not lyse human erythrocytes in the presence of non-electrolytes with hydrodynamic diameters of >5.0 nm, although it caused leakage of potassium ions and swelling of the cells under the same conditions(Tomita *et al*., 1998). Flammutoxin, assemble into a pore-forming annular oligomer with outer and inner diameters of 10 and 5 nm on the target cells. The electrophysiological properties of flammutoxin channels using planar lipid bilayer technique were studied, and found that the larger-conductance channel of flammutoxin corresponds to the haemolytic pore complex, while the smaller-conductance channel may reflect the intermediate state(s) of the assembling toxin(Gulnora *et al*., 2000) .

Cao *et al.* (1989) reported that PA3DE shown to have a MW of 5.4 x 106 which contained D-glucose, D-mannose and L-fucose in the molar ratio 22.31:1.46:1.00, and had β -glycosidic linkage. It showed inhibitory activity against the Sarcoma 180 tumor in mice.

A new fungal immunomodulatory protein (FIP-fve), having molecular mass of FIP-fve determined by SDS-PAGE agreed well with the value of 12 704 Da calculated

from its amino acid composition and sequence was isolated and purified from the edible golden needle mushroom. FIP-fve consisted of 114 amino acids residues with an acetylated amino end and lacked methionine, half-cystine and histidine residues. FIP-fve was able to haemagglutinate human red blood cells. The immunomodulatory activity of FIP-fve was demonstrated by its stimulatory activity toward human peripheral blood lymphocytes and its suppression of systemic anaphylaxis reactions and local swelling of mouse footpads. FIP-fve was found to enhance the transcriptional expression of interleukin-2 and interferon- gamma (Ko-Jiunn, 1995).

Tomita *et al*., (2004) cloned and expressed the cDNA encoding a 272-residue protein with an identical N-terminal sequence with that of Flammutoxin (FTX), a 31-kDa pore-forming cytolysin from *F. velutipes.* Ng and Wang (2004) investigated a protein designated as flammin and exhibiting a molecular mass of 30 kDa, and another protein designated as velin and possessing a molecular mass of 19 kDa, from the fruiting bodies of *F. velutipes*. Flammin and velin inhibited the translation in a rabbit reticulocyte lysate system with an IC50 of 1.4 and 2.5 nM, respectively. Flammulin, an antitumour protein, was purified from the aqueous extract of basidiomes of *Flammulina velutipes* to electrophoretic homogeneity, its molecular weight was 24 kDa, determined by SDS polyacrylamide gel electrophoresis (Zhou *et al*.,2003). Zhou et al. (2003) extracted mRNA of *F. velutipes* using Quick Prep mRNA Purification Kit for flammulin

Flammulina velutipes showed inhibitory effects on HP urease (Kim, 1996). A new protein that decreases transepithelial electrical resistance (TEER) in the human intestinal Caco-2 cell monolayer was found in a water-soluble fraction of the mushroom *F. velutipes.* This protein, termed TEER-decreasing protein (TDP), is not cytotoxic and does not induce cell detachment, but rapidly increases the tight junctional permeability for water-soluble marker substances such as Lucifer Yellow CH (Mr 457) through the paracellular pathway (Watanabe *et al*.,1999). The anti-complementary activity (immunostimulating activity) of endo-polymer was found to be 77% in *F.velutipes* (Song *et al.*,1998). Paaventhan *et al*. (2003) reported that Fve, a major fruiting body protein from *F. velutipes,* a mushroom possessing immunomodulatory activity, stimulates lymphocyte mitogenesis, suppresses systemic anaphylaxis reactions and oedema, enhances transcription of IL-2, IFN- gamma and TNF- alpha , and hemagglutinates red

blood cells. It appears to be a lectin with specificity for complex cell-surface carbohydrates. Fve is a non-covalently linked homodimer containing no Cys, His or Met residues.

Zhang (2005) observed that some polysaccharides from *F. velutipes* inhibited tobacco mosaic virus and cucumber mosaic virus infecting *Chenopodium amaranticolor*. An antiviral protein (Zb) was isolated and purified from *F. velutipes* by different chromatographic methods. SDS-PAGE showed that the protein has a molecular weight of ~30 ku (Fu *et al*.,2003). *Flammulina velutipes* significantly inhibited mycelial growth of the four phytopathogenic fungi (*Cochliobolus sativus*, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici*, and *Rhizoctonia cerealis*), responsible for foot and root diseases of winter cereals (Badalyan *et al.*, 2002).

Ishikawa *et al*. (2001) isolated two new cuparene-type sesquiterpenes, enokipodins C (1) and D (2), from culture medium of *Flammulina velutipes*, along with enokipodins A (3) and B (4). All the metabolites showed antimicrobial activity against a fungus, *Cladosporium herbarum*, and Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*. Wang and TziBun (2001) isolated a single-chained ribosome inactivating protein (RIP) with a molecular weight of 13.8 kDa from the fruiting bodies of the *F. velutipes*. The protein was novel in that it possessed a molecular weight lower than those of previously reported RIPs and that it was capable of inhibiting human immunodeficiency virus (HIV-1) reverse transcriptase β-glucosidase and β-glucuronidase.

Mitchell *et al*. (2007) demonstrated the anti-complementary activity for the exopolysaccharide of *Flammulina velutipes*. Two polysaccharides were isolated from the basidiomycete *Flammulina velutipes*, via successive hot extraction with water, 2% and 25% aq. KOH, and then submitted to freeze-drying (Smiderle *et al*., 2006). A hemagglutinin with a molecular mass of 12 kDa was isolated from the fruiting bodies of the mushroom *Flammulina velutipes*. Its molecular mass was similar to that of the fungal immunomodulatory protein isolated from *F. velutipes* (FIP-fve) with ice-cold 5% acetic acid and 50 mM 2-mercaptoethanol as extraction medium and to that of the larger 12 kDa subunit of *F. velutipes* lectin isolated with phosphate buffer as extraction medium (Ng *et al*.,2006). Transgenic lettuce lines containing the decarboxylase gene (oxdc) isolated

from a *Flammulina* sp. were produced by *Agrobacterium*-mediated transformation (Dias *et al*.,2006)

Physiological requirements:

In nature, the main substrates for mycelium growth are woody compounds, which are cellulose, lignin and monosaccharides. *Flammulina velutipes* grows and fruits on relatively simple natural media such as malt agar and potato-dextrose agar as well as on synthetic media. Ammonium compounds and amino acids are suitable nitrogen sources for the mycelial growth and fruit body formation. Inorganic nutrients such as Magnesium and phosphate are effective for the growth of of mycelium and for initiation of fruit body formation. The phosphate ions are reported to be indispensable for fruiting. The effect of various trace elements such as Fe, Zn, Mn, Cu, Co, Mo and Ca are also recognized. Among vitamins, thiamine has been reported to effect the mycelial growth and fruit body formation of *F. velutipes.*

Furlan *et al*. (1997) reported that mycelial growth rates were higher on WDA (wheat/dextrose/agar) medium than on PDA (potato/dextrose/agar) or MPA (malt/soya peptone/agar) media .Best growth of *F. velutipes* was observed in a medium containing 4% glucose as the C source, 0.2% peptone as the N source, 0.15% KH2PO4 and 0.05% MgSO4, and a pH of 6.0. The highest yield of mycelia was obtained when Zajiao 13 was cultured on this medium at 25^{o}C for 8 days (Su *et al*., 2001).

F. velutipes grew in the waste water from the starch industry as submerged culture and growth further improved with modifications to the nutrient composition of the medium (Zhu *et al*., 1997).Boyle (1998) reported that lignin degradation is important for growth and observed that N-availability limits growth rates of *F. velutipes* on wood while availability of simple carbohydrates, micronutrients or vitamins does not. Wang (2000) recorded that *F. velutipes* grew well in medium with soyabean powder, peptone, beef cream and yeast powder as N sources but could not grow well with nitrate or amine nitrogen as the N source. Yatohgo *et al*. (1988) purified a lectin which was dimeric acidic protein consisting of 2 identical subunits with an apparent molecular mass of 11 kD, with a pI value of 5.4, and devoid of cysteine, methionine, and histidine as amino acid constituents.

Genetics and breeding

Morphological analysis, mating studies and analysis of ribosomal ITS sequences were used to determine that a number of new species were hidden within the epithet *Flammulina velutipes* including *F. populicola* (growing on *Populus* roots), *F. rossica* and *F. elastica*) For true *F. velutipes*, data suggest that the center of origin was Asia, spreading to Pacific North America via the Bering land bridge and to Europe and Eastern North America, possibly via the North Atlantic land bridge. Hybridization was suspected in this group based on morphology and the ability of some species to interbreed *in vitro*, and was confirmed by finding a hybrid ITS sequence in *F. velutipes.*

The life cycle of *F.velutipes* is little different from other basidiomycetes since monokaryotic fruit bodies are formed and dedikaryotization is displayed. It can produce both monokaryotic as well as dikaryotic fruit bodies, however , haploid fruit bodies are smaller than dikaryotic fruit bodies with poorly developed pilei and few basidiospores. *F.velutipes* is tetrapolar and has four mating types. Dikaryotization occurs where both A and B alleles differ. All combinations of monosporous isolates are dikaryotized among strains having different incompatibility factors. Dikaryotic mycelia possessing different physiological and morphological charcterstics from their parental dikaryons can be obtained by crossing many monosporous isolates. From these dikaryotic mycelia suitable strains for commercial cultivation can be obtained.

Tissue Culture

Zheng *et al*. (2001) inferred that there was significant genetic stability in culture characteristics between the parental strain and isolated strains, and among the isolated strains from tissues of different parts of fruit body. Contrary to this Zhang-Gong *et al.* (2001) reported that he tissues from middle stipe of *F. velutipes* fruit bodies cultured on three media (I. potato extract 20% (w/w), glucose 2%, MgSO4 0.015% and KH2PO4 0.02%, II. potato extract 10%, mushroom stipe extract 10%, sugar 2% and agar 2%, and III.maltose 2%, peptone 1%, yeast juice 1% and agar 2%) media had hyphal growth potential) or hyphal growth velocity values higher than those from juncture of pileus and stipe, whereas the tissues from basal stipe on III media didn't grow. The tissues from any part of *F. velutipes* on medium III had hyphal growth velocity or hyphal growth potential values higher than those on I and II. Magae *et al*. (2005) developed a simple colorimetric method to detect degenerated strains by using a liquid medium supplemented with

bromothymol blue and lactose. The ability of a strain to develop normal mushrooms could be determined by the colour of the medium.

Protoplast fussion

Yamada *et al*. (1983) used protoplast fussion to develop new strains and observed that the mycelium age (2 to 24day-old cultures) greatly influenced protoplast isolation. The highest yields of protoplasts (6.7 x 10-7 cells/ml) were obtained from 2 to 3 day-old mycelial cultures. Protoplast regeneration was most rapid on OS (onion juice + soy sauce). medium. Zhao and Chang (1993) discussed the applications of proplast fussion in comparison with other monokaryotization methods for the genetic improvement and observed that protoplasting is rapid, simple and effective.

Breeding through Oidia

Yu *et al*.(1998) determined the occurrence, germination, nuclear behaviour and polarities of sexuality of oidia from different isolates of *F. velutipes*. It was observed that both monokaryons and dikaryons could give rise to oidia. Most oidia were cylindrical or oval in shape, and only a few were round or Y-shaped. Oidia germinated easily, and the germ tube was usually wider than the oidia. Oidia produced by monokaryons were all uninucleate and had the same sexuality as the parental mycelium. Oidia from dikaryons were also uninucleate. Binucleate or multinucleate oidia were not observed. A portion of the oidia produced by dikaryons had the same mating type as one of the parental mycelia, while the other portion had the same mating type as the other parent. Zhang *et al.(*1990) used oidiospores for making crosses between varieties. No significant differences in hyphal growth rate, fruiting capacity, yield and quality were found between crosses involving oidiospores and those involving basidiospores. The use of oidia was simpler and more rapid since they could be obtained more easily than basidiospores.

Markers

Isoenzyme markers of hybrids from 4 crosses between different strains of *F. velutipes* were analysed by PAGE (Hu *et al*.,1997) . All the hybrids resulting from these crosses had zymograms different from those of their parents. The 2 biochemical markers EST (esterase) and PRO (soluble protein) showed complementary effects that resulted in the presence of new bands in hybrids. Hybrid heterosis could therefore, be predicted at an early stage using zymograms combined with the number of marked heterozygous loci.

Yun (1996) reported that brown and white strains also responded similarly to the various pretreatments applied (alternating temperature (the best treatments were 5-10 and 5-15^{o} C), ultraviolet radiation for 30-50s, electric current for 5s, thiamine hydrochloride (0.05-0.1%), urea and IAA). The brown strain had a larger pileus and longer stipes than the yellow strain, while the yellow strain produced more biomass and a greater number of fruit bodies. Xie *et al*. (2004) identified the allele for the white colour through, a pair of sequence-characterized amplified region (SCAR) primers. The SCAR primers amplified the unique fragment for all strains that carry the allele for the white colour of the fruiting body of *F. velutipes*. The products of polymerase chain reaction analysis of the SCAR primer could be detected directly under an ultraviolet lamp; electrophoresis was not always necessary. Zhu *et al*. (2007) evaluated the germplasm resources of *F. velutipes* using sequence-related amplified polymorphism (SRAP) to study the genetic difference between yellow and white strains. Thirty tested strains were classified into 14 categories. SRAP analysis was efficient, stable and repeatable, and could be used in the construction of the genetic linkage map, identification of strains and molecular marker-assisted breeding. Esterase isozymes from *F. velutipes* showed many bands and variations among the different stocks on the gel. The stocks of *F. velutipes* in Japan were classified into three groups (A, B, and C) according to the cluster analysis of esterase isozymes. Group C was characterized by a larger spore size, slower spawn running, and a paler pileus color than groups A and B. Furthermore, group B showed a smaller spore size, slower spawn running, and paler pileus color than group A (Nishizawa *et al*., 2003).

Radiation

Jin *et al*. (2000) developed a high yielding strain using 60 Co- gamma ray treatments indicating that protoplast radiation induction is an effective method for *F. velutipes* breeding.

Preservation of cultures

Kitamoto *et al*. (2002) demonstrated that the sawdust-freezing method using a cryoprotectant is a reliable and easy preservation method for fungus stock cultures of *F. velutipes*. Five disks of *F. velutipes* kept in a vial containing 1 of 3 cryoprotectants: 10%

glycerol, 5% DMSO or 10% polyethylene glycol and stored for 7 years at -20^{o}C, -85 ^{o}C or liquid nitrogen temperature revealed that four mycelial disks preserved at -20 ^{o}C showed higher yields than those preserved at the other temperatures (Ohmasa *et al.*,1996). Among the cultures derived from strain FMC224, the control cultures preserved by subculture showed the lowest yield.

When *F. velutipes* was cultured on ordinary SCM, xylanase, CMC and amylase activity peaked on day 6, 4 and 3 of culture, respectively. When *F. velutipes* was cultured on carbon-source-changed SCM, amylase activity peaked twice, on day 3 and 5 of culture, and xylanase and CMC activity peaked on day 5 of culture. Protein and RS contents changed over time on both media and were related to enzyme activities. Protein and RS contents and the total activity of the 3 enzymes were higher on ordinary SCM than on the carbon-source-changed SCM (Li *et al*., 2002).

Spawn preparation

Saw dust spawn: The sawdust of *Eucalyptus* sp. (80%) and rice bran (20%) are used for the spawn preparation. The mixture is adjusted at the moisture of 60% (Yang, 1986) and then filled in the glass jar of 500 ml capacity. After autoclaving at121^{o}C for 1 h, the spawn medium is inoculated with bits (one disc of one cm in diameter) of mycelia of strain growing vigorously in PDA slants and then incubated at 24^{o}C in dark. The spawn in the jars becomes ready for inoculation to the substrate after 20 days growth when the mixture turned totally white.

Grain spawn: The boiled wheat grains are filled into half litre glucose/milk glass bottles upto 3/4th of the capacity of the bottles. Bottles are plugged with non-absorbent cotton and are sterilized at 22 lbs p.s.i. (126C) for 1.5-2 hours. Sterilized bottles are taken out from the autoclave while still hot and are shaken to avoid clumping of the grains. These bottles are allowed to cool down overnight to room temperature. Polypropylene bags (12.5x27.5 cm) accommodating about 250-260 g of spawn in each bag can also be used for the sake of convenience in transportation. About 40-50 bags are inoculated from one master culture bottle. The bags after inoculation are incubated at 25±1°C. In two weeks after inoculation, spawn bags are ready for use.

Cultivation in India

Sawdust and rice bran / wheat bran are commonly used as substrates for cultivation. Saw dust of broad tree leaves is required for the cultivation of this mushroom. Sawdust media have oxygen and water which is necessary for mycelial growth. Rice bran/wheat is used as a supplement which provides many of the essential nutrients. Much of the lignin, cellulose, and monosaccharides are provided by the sawdust. Saw is wetted thoroughly with water for 16-18 hrs. Generally, equal quantity of saw dust is poured in equal quantity of water in 100lts capacity tubs. After wetting 5 per cent wheat bran is added in the saw dust and mixed thoroughly.

Containers: This mushroom can be grown in a variety of containers like Polypropylene bags, plastic bottle, vinyl bag, filter bag and jars (Fig.1.)

Filling: Polypropylene bags (2000 g) are used for the cultivation. Two kg substrate (soaked) was filled in each bag. The bags are plugged with non absorbent cotton by inserting a ring in the mouth of the bag.

Sterlization: The filled bags are sterilized in the autoclaves for 1½ hour at 15 Ibs pressure per square inch.

Inoculation: After the bags have been sterilized and cooled down to 20°C, they are inoculated with wheat grain based spawn. Saw dust spawn can also be used which growers may purchase from specialist spawn makers. Sawdust spawn is prepared by mixing ten parts of saw-dust with one part of rice bran and enough water to provide a certain degree of humidity.

Incubation (Spawn run):The Bags are placed/ arranged in incubation rooms where mycelia can grow favorably. The optimum temperature for the mycelial growth is between 22 and 25°C, so the temperature of incubation room is kept between 20°C and 23°C under the normal commercial cultivation conditions. Mycelia spread over the whole bag after 20-25 days (Fig.2).

Fig.1: Different containers used for the cultivation of winter mushroom

Fig.2: Spawn run bags

Fig.3: Bags ready for fruit bodied induction

Fruiting induction:

When mycelial spread to 90% of the bag space, the plug is pulled off, the neck of the bag is unfolded and the surface of the media is made smooth for fruiting (Fig.3). Bags are then placed in the dark at a temperature of 10 -14 ℃ and the humidity is maintained at 80

Fig.4: Initiation of primordia **Developing primordia**

-85%. A moisture level in the bags is important to fruiting. Good fruit bodies are encouraged to form by adjusting the humidity in the room to maintain the correct moisture content of the substrate. Primordia are formed in 10-14 days after reducing the temperature(Fig.4).

Controlling: At 10 -12°C, the fruit bodies grow rapidly, but they are slender, long, and of poor quality (Fig.5). For this reason, the growth of fruit bodies is controlled by

lowering the temperature to 3-5°C and providing air movement (3-5m/sec) which provide stiff, white, and drier fruit bodies. This control is continued for 5-7 days, from the period when the cap's differentiation is observed with the naked eye to the period when the length of the stem reaches 2 cm.

Fig.5: Slender, long fruit bodies

Cropping: When the fruit bodies are from 13-14 cm, the rolled paper is removed and fruit bodies are pulled up from the bottle and packed. It takes about 50-60 days from the initial fruiting to the crop. The first crop usually amounts to 100- 140 gm/800 ml bags and the second crop from 60-80 gm in the same bag (Fig.6). Bottle/Jar culture is not recommended for commercial cultivation (Fig.7). Kachroo (1991) obtained increased yield by dipping the bags in a solution of copper (0.04mg), Fe (0.02mg) and (5.0mg) after spawn run. Sharma (2004) cultivated winter mushroom by filling 2 Kg wet substrate (mixed saw dust) in Polypropylene bags, sterilized at 121^{o}C for 2 hrs, inoculated and incubated at 25 ^{o}C. After the completion of spawn run temperature was lowered to 15C. After lowering the temperature, fruit bodies appeared in 18 days which became ready to harvest in the 6 days. A biological efficiency of 45 per cent was achieved.

Fig. 6: Cultivation in polypropylene bags

Fig.7:Cultivation in jars

Management: The initiation of fruit bodies starts in dark but light is necessary for the further development. At the time of fruit body formation, temperature of cropping room should be lowered to 8-12C with relative humidity from 80-85 per cent.

Harvesting: When the fruit bodies are 14-18 cm long, the fruit bodies are harvested (Fig8). They are packed in PP bags or can be sun dried. After harvesting second flush appears in about 15 days. Only two flushes are harvested. About 360-400 g fresh mushrooms can be harvested per bag containing 2 Kg wet substrate thereby giving 36-40% B.E.

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Fig. 8: Crop ready to harvest

Cultivation in other countries

Substrates: Various substrates and cultivation procedures tried for winter mushroom cultivation is as under:

The mushroom is cultivated in a plastic bottle or a vinyl bag or polypropyene bags for 30 days at 20°C and 70% humidity, on a substrate of saw dust or corn cobs, and a number of additional ingredients. Afterwards, the mushroom is grown for another 30 days in a slightly cooler but more humid environment. The growth is constricted to force the mushroom to grow long and thin. The mushroom available in the supermarket often still shows the impression of the bottle around the base of the mushroom.

Flammulina velutipes has been cultivated on wood logs or on saw dust medium. Since white, stiff, and durable sporocarps are preferred, now-a day's cultivation on sawdust is common. The quality of the *F. velutipes* cultivated on wood logs was inferior hence presently its cultivation is mainly done on saw dust.

Production of most enokitake is based on synthetic substrate contained in polypropylene bottles. Substrates (primarily sawdust and rice bran; 4 : 1 ratio) are mechanically mixed

and filled into heat resistant bottles with a capacity of 800 to 1,000 ml. Sawdust consisting primarily of *Cryptomeria japonica*, *Chamaecyparis obtusa* or (aged 9 to 12 months) *Pinus* spp. appears to offer the best yields. In the United States, a bran-supplemented medium, consisting primarily of corn cobs, serves as the primary medium. After filling into bottles, the substrate is sterilized (4 h at 95°C and 1 h at 120°C), mechanically inoculated and incubated at 18° to 20°C for 20 to 25 days. When the substrate is fully colonized, the original inoculum is removed mechanically from the surface of the substrate and the bottles may be placed upside down for a few days. At the time of original inoculum removal, the air temperature is lowered to 10° to 12°C for 10 to 14 days.

To further improve quality during fruiting, temperatures are lowered to 3° to 8°C until harvest. As the mushrooms begin to elongate above the lip of the bottle, a plastic collar is placed around the neck and secured with a Velcroreg. strip. This collar serves to hold the mushrooms in place so that they are long and straight. When the mushrooms are 13 to 14 cm long, the collars are removed and the mushrooms are pulled as a bunch from the substrate. The mushrooms then are vacuum packed and placed into boxes for shipment to market.

San-Antonio and Hanners, (1983) cultivated *F. velutipes* successfully on wood logs using grain-spawn disk inoculum. Miller (1998) also used logs and sawdust, for the cultivation of *F. velutipes* mainly in China and Japan.

Gavrilova and Lysenkova (1988) tried various substrates consisting of *Populus tremula*, sawdust + wheat straw (10:1), sawdust + wheat straw + spent malt (10:1:1), and sawdust + spent malt (10:1), produced yields equal to 15.5, 19.8 and 20.9% of the weight of the substrate, respectively. Cellulose, lignin and carbon utilization from the substrates during growth and fruiting revealed that the initial 0.17-0.41% N concentration of the substrates rose to 0.38-0.80%. Schmidt, (1985) also described optimum culture temperatures, pH, nutrient media and nitrogen sources for *F. velutipes*.

Dang-Jian *et al*. (2001) conducted tests to investigate the effects of fermentation period, culture material (CM), height and carbendazim concentration in the medium on the growth and physiology of *F. velutipes* cultivar (F801 strain). In test I, cotton seed hull medium was supplemented with carbendazim at 0, 0.1, 0.15, 0.2 and 0.25% to prepare media (1), (2), (3), (4) and (5), respectively, of which medium (1) was sterilized, but the others were not. When F801 was inoculated on media 1-5, the carbendazim inhibited hyphal growth, and this inhibition increased with increases carbendazim concentration. In test II, medium 1 was fermented at 12^{o}C, and sampled after 48, 72, 96 or 120 h. The highest fresh mushroom yield was obtained on medium sampled after 96 h of fermentation. In test III, the medium fermented for 96 h was bagged to heights of 10, 8, 6 or 4 cm, then inoculated with F801 strain. The highest biological conversion efficiency 112% was found on the CM with a height of 8 cm.

Payapanon and Tontyaporn (1999) studied the effects of heat treatments (100^{o}C for 4 h (A), 100^{o}C for 3 h/121 ^{o}C for 1 h (B) or 121^{o} C for 1 h (C)), used to sterilize substrate media (rubber sawdust alone or in combination with rice bran, corn flour or water, 800 g in polypropylene bags), on contamination of *F. velutipes* cultures were investigated. Two flushes were harvested from each treatment. For the first flush, contamination was in the range 0-4, 15-25 and 50-80% for heat treatments A, B and C, respectively. For the second flush, low contamination and high production rates were observed in treatments in which the substrate media were heat treated at 100^{o} C for 4 h.

Ding *et al.(*1990) reported that the use of plant growth regulators including alkylal 30, gibberellin, alkylal 30 + gibberellin, ethylene, kinetin, and methyl α -naphthyl acetate during different growth periods of *Flammulina velutipes* shortened the time to appearance of fruiting bodies, and increased the number, yield and quality of the fruiting bodies. Alkylal 30 + gibberellin gave the best results.

The fruit-bodies were much heavier in flowing air, mainly because of greater pileus size. Young fruit-bodies left singly on a mycelium grew larger than those left in groups; they attained 60-80% of the total weights of groups of 3-7 fruit-bodies each in still air and 54-64% of the weight of 5 fruit-bodies in the flowing air. Fruit-bodies of the same rank weighed more in smaller than in larger groups, but the total weights of groups of 3-7 fruit-bodies each did not differ significantly. There was also competition between

fruit-bodies in their late phase of rapid elongation. Pileus/stipe weight ratios increased with decreasing numbers of fruit-bodies on a mycelium and were greater in flowing than in still air. The results indicate that fruit-bodies in groups inhibit each other's growth by competing for materials, required for growth, which are supplied in limited amounts from the mycelium and distributed among individuals proportionately to their ranks (Gruen, 1983).

Zadrazil (1980) described different containers suitable for growing edible fungi and observed that plastic container is the best for *F. velutipes*. Growth of fruiting bodies was better on the sides of a cylindrical container than on a flat surface. Various types of apparatus for constructing cylinders and blocks of substrate, and for providing them with aeration and warmth while the crops are produced. A new method of bag cultivation for *F. velutipes*, regarded as regeneration cultivation, is introduced (Cai,1989). Park *et al*. (1978) recorded optimum yields of fresh material of *F. velutipes* in 800 ml-flasks containing 660 g poplar sawdust to which rice bran was added at 40% by volume and adjusted to 70% moisture content, with a growing temperature of 6^{o}C. *F. velutipes* was grown in glass jars on 50 g damp wheat straw alone or enriched with 20 g soaked crushed bean, pea or wheat seeds, or on 100 g damp wood shavings alone or similarly enriched. It fruited better on supplemented wheat straw (Hubsch, 1983).

Gramss(1981) grew *F. velutipes* using substrates containing beech sawdust, wheat straw, wheat flour, wheat bran and pea haulm. The fungus needs a substrate with high carbohydrate and N contents, each in an easily available form. *Acacia mearnsii* sawdust proved to be a good substrate for growing *F. velutipes* (Lin, 1991). Chu-Chou (1983) cultivated winter mushroom on sawdust from New Zealand timbers. Gramss,(1977) cultivated *F. velutipes* on blocks made of mixtures of sawdust, clover, hay, dried pea haulm, dried lucerne, wheat flour, sugar and other materials. Pawlak and Siwulski (2001) evaluated yield of two *F. velutipes* cultivars (F-01 and F-04) on sawdust cultivation media consisting of sterilized pine or beech sawdust as well as a mixture of both types. They observed that cultivation media influenced the yield of *F. velutipes.*

Yoshizawa(1998) reported that sawdust prepared from smoke-heated logs of sugi and karamatsu, the hot-water, 1% sodium hydroxide, and ethanol-benzene extracts contamination were reduced in comparison with non-treated sawdust. Poppe and

Heungens (1991) evaluated ten woods (*Acer, Alnus, Betula, Carpinus, Fagus, Fraxinus, Picea, Pinus, Populus, Salix*) as substrates for *F. velutipes*.

Tang (1988) described the cultivation of *F. velutipes* in plastic sheds, either in bags or in boxes on a substrate consisting of 95% cotton seed hulls. Boxes gave better yields. A high humidity of 85-95% should be maintained, it is recommended that the sheds should be kept open for 2 h each daily. Early harvesting, when the fruiting bodies reach a length of 13-15 cm should be done for better quality fruiting bodies. Miao(1988) cultivated *F. velutipes* in beds of cotton seed shells and highly inconsistent results were obyained the three crops he raised. Zhao (1989) described the cultivation of *F. velutipes* underground in jars on a substrate of 93% cotton seed hulls, 1% sucrose, 1% gypsum and 5% ground maize. Illumination was provided by 15-25 W incandescent lamps spaced 25 m apart. A temperature of 13-15^{o}C and a high humidity of 80-90% favoured growth. With this system the average yield was 0.197 kg/jar.

Xiong *et al.*, (1999) compared the growth of *F. velutipes* on media containing 88% cotton seed hulls, 1% gypsum and 1% refined sugar with 10% quail droppings (aged for 0-40 days). It was observed that growth rate increased with aging of droppings up to 30 days, and then decreased slightly with aging for 40 days. Average yields were 178 g/jar with fresh droppings, increasing to 219 g with droppings aged for 30 days and 214 g with droppings aged for 40 days. In another combination consisting of 78-93% cotton seed hulls, 5-20% quail droppings, 1% gypsum and 1% refined sugar the growth was most rapid with 10% droppings and slowest with 20% droppings. Average yields were 193, 213, 199 and 158 g/jar with 5, 10, 15 and 20% droppings, respectively.

Application of vitamins to the growing medium increased hyphal growth and productivity of *F. velutipe*. Chen-Hai *et al.*,(1995) obtained high productivity and good quality mushrooms using a substrate of cotton hull locules (50%), rice bran (20%), wheat bran (15%), corn powder (10%) and 5% bean cake, whereas Wang (1989) observed that the best culture medium for the cultivation was the medium comprising of cotton seed husks (88%), wheat bran (10%), vitamin B1 (0.2%), vitamin B2 (0.3%), MgSO4 (0.5%) and CaSO4 (1.0%). Li (1989) grew four strains of *F. velutipes* on substrates consisting mainly of cotton seed hulls or unsterilized maize cobs. He observed that the period between inoculation and appearance of the fungus was longer on maize cob substrate

than on cotton seed hulls, but the yield was from 2.9 to 9.6 times higher on maize cob substrate than on cotton seed hulls, depending on the strain of the fungus. Yang-Xiao (2001) used three types of media with different carbon sources and revealed that there was no significant difference in the yield of *F. velutipes* between the medium of fir sawdust mixed with crust of cottonseed and the medium of sole cottonseed crust. However, the medium of fir sawdust was more practical due to its its low cost.

Lu *et al*.(1989) cultured *F. velutipes* on 7 media: 1. 80% distillers' barley + 20% cotton seed husk; 2. 40% distillers' barley + 60% cotton seed husk; 3. 88% cotton seed husk + 10% broiler concentrates + 1% sugar + 1% lime; 4. 100% cotton seed husk (control); 5. 89% cotton seed husk + 10% broiler concentrates + 1% lime; 6. 98% cotton seed husk + 1% sugar + 0.5% urea + 0.5% lime; and 7. 89% rice straw + 10% concentrates + 1% lime. The highest mycelial growth rate, 39 cm/day, was achieved on the last medium (7) and the lowest rate, 24 cm/day, on the first medium (1). The harvest times for the first crop of mushrooms were approximately the same, but they varied significantly for the 2nd harvest (31-37 days). The biological efficiency was the highest, (98.6%), in medium 3 and lowest (50.9%), in medium 7, with the order (3) > (2) > (6) > (1) > (5) > (4) > (7).

Ji-Hong *et al*.(2001) inoculated *F. velutipes* on 4 media, namely, (1) 70% maize straw (MS), 10% cottonseed hulls, 15% wheat bran (WB) and 5% maize flour (MF), (II) 88% MS, 5% WB, 5% MF, 1% sucrose and 1% lime, (III) 90% MS, 5% WB and 5% MF, and (IV) 79% MS, 5% WB, 5% MF, 10% sawdust and 1% lime. The best media formulae for hyphal growth of F. velutipes and G. lucidum were II and IV, respectively. Jinzha No. 19 a cultivar of *F.velutipes* was the best strain with biological efficacy of 73% on medium II.

Tang-Xiang *et al*.(2001) evaluated four media comprising of flour formulae, (1) 80% rice straw, 10% rice bran, 7% corn flour, 2% plaster, 1% sucrose; (2) 80% *P. notatum*, 40% cotton seed hulls; (3) 92% cottonseed hulls, 5% rice bran, 1% calcium superphosphate, 1% plaster and 1% urea, and (4) 90% *P. notatum*, 6% rice bran, 2% calcium superphosphate, 1% plaster and 1% sucrose. These media were sterilized for 1.5 h at 126^{o} C and inoculated with *F. velutipes*. The hyphal growth rate on the 4 media was ranked 4<2<1<3. The highest biological conversion rate was recorded on formula C (106.68%),

next highest on formula B (90.61%), the third highest on formula D (80.76%), and the lowest on A (76.73%). The input output ratios of formulae A, B, C and D were 1:1.86, 1:2.17, 1:2.08 and 1:2.03, respectively.

Thielke(1989) cultivated *F. velutipes* successfully on coffee grounds, a waste material produced in the manufacture of coffee powder. Arai *et al*. (2003) reported hot water-soluble fraction (HWSF) from corn fiber (CNF) an abundant by-product of the wet corn milling process, can be used as a promotive substance for cultivating *F. velutipes*. Gao *et al*. (1992) gave preliminary report on the use of feather meal in the cultivation of the winter mushroom.

Containers: This mushroom can be grown in a variety of containers like Polypropylene bags, plastic bottle or a vinyl bag , filter bag and poly propyene.Commercially, winter mushroom is grown in jars The mushroom has been cultivated in Japan initially on wood, and later in the bottles.

Solid state cultivation (SSC) *F.velutipes* is cultivated on coffee spent-ground or coffee husk which is also known as solid state cultivation (SSC). SSC is carried out using substrates filled in plastic bags (20x35 cm) filling100 g substrate in each bag on dry wt basis (Fan *et al*.,2001). These substrates are moistened with water (60% and 55% for coffee husk and spent-ground, respectively) generally 4-5 h before autoclaving and are autoclaved at 121^{o}C for 1.5 h. On cooling these bags are inoculated with spawn @10% and mixed thoroughly to facilitate rapid and uniform mycelial growth. Higher spawn rates viz. 25% did not give significant higher yields. The mouth of bags are sealed using a cotton plug or thread. Then these bags are incubated in the dark at 24^{o}C. Coffee husk and spent-ground without any nutrients supplementation has been used for cultivation of *F. velutipes* LPB 01 in solid state cultures. Synthetic substrate consisting of primarily sawdust and rice bran in the ratio of 4 : 1 is being also used for the cultivation of winter mushroom in Japan. Sawdust consisting primarily of *Cryptomeria japonica*, *Chamaecyparis obtusa* or aged (9 to 12 months) *Pinus* spp. appears to offer the best yields.

On coffee husk substrate, first fructification occurred after 25 days of inoculation and the biological efficiency reached about 56% with two flushes after 40 days. On

spent-ground as substrate, first fructification occurred 21 days after inoculation and the biological efficiency reached about 78% in 40 days. There was decrease in the caffeine and tannins contents (10.2 and 20.4%, respectively) in coffee husk after 40 days. In coffee spent-ground, the tannin contents decreased by 28% after 40 days. This decrease was attributed to the degradation of caffeine or tannins by the culture because these were not adsorbed in the fungal mycelia. Results showed the feasibility of using coffee husk and coffee spent-ground as substrate without any nutritional supplementation for cultivation of edible fungus in SSC. Spent ground appeared better than coffee husk.

Xing *et al*., (2007) studied the effects of adding As, Hg, Pb and Cd to compost on the yield and heavy metal enrichment of fruit bodies of *F. velutipes.* The results showed that the As, Hg, Pb and Cd contents of fruit bodies of *F. velutipes* increased with an increase in heavy metal concentrations of compost. Hg decreased the biological efficiency, but Cd slightly improved the biological efficiency. The regression analysis showed that there was a significant linear correlation between the heavy metal concentrations of compost and the corresponding contents of the fruit bodies. According to the safe limits of heavy metals of the national agricultural standard 'Green Food: Edible Fungi' (NY/T 749-2003). It was concluded that the safe limits of As, Hg, Pb and Cd in compost of *F. velutipes* is lower than 1.0, 0.1, 10.0 and 1.0 mg/kg, respectively. Zou, *et al*. (2005) revealed that growth-promoting agents accelerated hyphal growth of the fungus and increased its yield. An optimum combination was also identified, which shortened vegetative growth period by 4.6 days and increased the biological efficiency by 55.08%.

In the United States, a bran-supplemented medium, consisting primarily of corn cobs, serves as the primary medium. After filling into bottles, the substrate is sterilized for 4 h at 95°C and 1 h at 120°C, inoculated mechanically and incubated at 18° to 20°C for 20 to 25 days. When the substrate is fully colonized, the original inoculum is removed mechanically from the surface of the substrate and the bottles may be placed upside down for a few days. At the time of original inoculum removal, the air temperature is lowered to 10° to 12°C for 10 to 14 days (Royse, 1996).

Effect of moisture and spawn rate: Substrates were prepared with different moisture levels namely 45, 50, 55, 60, 65, and 70% for SSC. Similarly, different spawn rates were tested, which included 2, 5, 10, 15, 20, and 25%. After the 20 days fermentation, the protein and fibre contents in the substrate were measured. Moisture contents 55-60% and spawn rate 10% were the best.

Effect of environmental factors on the production

Age of the mycelial culture is very important in determining the fructification. It is difficult to form fruit bodies if the culture is too young (less than 5 days) or too old (older than 2-3 weeks duration).The optimum temperature for the mycelial growth is between 22^{o}C and 26 ^{o}C. The mycelium grows slowly, but does not die, when exposed to a temperature of 3 ^{o}C – 4 ^{o}C. On the other hand, at around 34 ^{o}C growth ceases and at over 34 ^{o}C the mycelium is killed for a short time. The temperature necessary for primordial formation is between 10 and 20 ^{o}C. The fructification induced more quickly at a temperature of 15 ^{o}C than 5 or 10 ^{o}C. Optimum temperature for fruit body growth is almost the same as that for primordial formation.

Moisture, humidity and temperature are indispensable factors for growth of mycelia and fruit bodies formation. It was found that mycelia grow most quickly in the presence of 60-65% water. *Flammulina velutipes* is aerobic and, accordingly, must be supplied with sufficient oxygen. It has been observed that pileus diameter decreased with the increasing concentration of CO_2. Stipe elongation has been found to be less sensitive to CO_2 than pileus expansion and stipe elongation as well as pileus expansion are both prevented by high concentration of CO_2.

Primordia of *F. velutipes* are induced in the dark condition, but light is essential for the maturation of the fruit body. Without light, only rudiments of a fruit body are formed.

Sakamoto *et al.* (2002) investigated the influences of temperature on the fruit body of *F. velutipes* and revealed that the fruit body could be induced after reducing of the ambient temperature under complete darkness, but could not be induced after light irradiation without temperature reduction. Fruit bodies formed under complete darkness elongated without pileus formation (pinhead fruit body). Pihakaski (1977) grew *F.*

velutipes on liquid nutrient medium, first in darkness at 25 ^{o}C for 7 days and thereafter in continuous light at 15 ^{o}C . Fruit body primordia were visible after 10-11 days from the start of the light period. Experiments on the commercial cultivation of *F. velutipes* in beds showed that a reasonably high CO_2 concentration was a key factor in the control of cap opening and growth promotion, and that low light conditions induced elongation towards the light. The response of *F. velutipes* to light under CO_2 concentrations of 0.114-0.152% was used to improve the yield and quality of fruiting bodies (Zhao,1990). Kaneko and Sagara (2001) revealed that fruit-bodies of some lignicolous agarics grew straight downward. *F.velutipes* fruit-bodies showed almost the same behaviour as the 'Non-Coprinus Type. Based on these results, gravitational responses in hymenomycetes are overviewed.

Sakamoto *et al*. (2004) showed that fruit bodies of *F. velutipes* could be induced in complete darkness after a sharp temperature reduction from 23^{o} to 16^{o}C. However, the fruit bodies that form in complete darkness have a long stipe with an undeveloped pileus on the top (pinhead fruit bodies) and are thinner and whiter than the normal fruit bodies which are formed in the light. This finding suggests that *F. velutipes* fruit bodies cannot mature in complete darkness. Sakamoto *et al.* (2007) identified a cell wall-associated protein (PSH) that was specifically induced in the pileus, but not in the stipe, following light treatment of the pinhead fruiting body. Cloning and sequence analysis of the gene encoding PSH (psh) revealed a motif in the C-terminal region of the predicted amino acid sequence that was similar to hydrophobin. The level of transcription of psh was low in the stipe, but it was expressed at a high level in the pileus of the normal fruiting body. Transcription was also low in pinhead fruiting bodies, but increased after light treatment. These results indicate that psh is specifically expressed during pileus differentiation induced by light stimulation.

Post harvest management

The mushroom are sold as fresh or canned, the fresh mushroom being preferable. Cut off the root system (approximately 4 cm) and wash briefly before use. They are traditionally used for soups, but can also be used for salads and other dishes. They have a fruity flavor and a crisp texture. The mushroom can be refrigerated for about one week.

Shelf life of *F. velutipes* is approximately 14-20 days at 1^{o}C, 10 days at 6 ^{o}C and 2-3 days at 20 ^{o}C . Browning of the pilei, gills and stipes and polyphenoloxidase activity markedly increased during storage at 20 ^{o}C. Total free amino acid content also greatly increased at 20 ^{o}C (Minamide *et al*., 1980). Fluctuating temperature had a particularly marked effect on the quality of this mushrooms (Ito and Nakamura ,1985).

The effect of packing materials (polyvinylidene chloride coated, oriented nylon, antifogging, wrap or vacuum packing film) on the keeping freshness of *F. velutipes* at 25 or 2 ^{o}C were investigated. The best treatment for maintaining quality was packing in antifogging film; *F. velutipes* could be stored for 28 days at 2 ^{o}C (Chi ,1998).Low-density polyethylene (LDPE) film embedded with 30 micro m (AC30) or 60 micro m (AC60) of silver-coated ceramic and used as packaging film for enoki mushrooms revealed that, AC30 had a storage life of 14 days compared with 10 days when packaged with LDPE without ceramic at either 5 or 20 ^{o}C (Eun *et al*.,1997). The effects of temperature (0 or 6 ^{o}C) and packing (sealed in polyethylene or sealed in polyethylene with a hole, alone or in combination with corrugated cardboard) on the shelf life of *F. velutipes* revealed that the best treatment was packing in sealed polyethylene and storing at the lower temperature; shelf life was extended to 15 days (Chi *et al*.,1996).

Wang and Xu (1992) designed an experimental drier used for thin layer drying studies of *F. velutipes* (Sing). Data were obtained for 8 drying conditions involving wind speed of 1-3 ms-1,temperature of 40-70 ^{o}C and RH of 16-30%. Two mathematical models, a diffusion model and the Page equation, were used. The Page equation was found to be more suitable for describing the drying characteristics of a single layer of *F. velutipes.* From an analysis of the drying rate, energy consumption and product quality, a temperature of 48^{o}C and wind speed of 2 ms-1 were found to be the optimum conditions.

F. velutipes stored in air at 5, 10, 20, 30 or 35 ^{o}C for 60 h, and exposed to ethylene at 100 p.p.m. for a 24-h period starting 12 h after storage showed no response to ethylene at any temperature (Inaba *et al*.,1989). The quality of mushroom, as affected by vacuum packaging, packaging unit and shelf temperature, was investigated during simulated shipping and marketing. Vacuum packaging of a 200-g-unit mushroom seemed to have higher potential for quality maintenance until the 14th day of shipping, while no

significant quality changes were observed between vacuum- and non-vacuum packaging after 21 days of shipping(Park *et al*.,2003).

Fresh mushrooms (100 g) were packaged under various conditions and stored at 10 ^{o}C for 14 days. The half-vacuum package was best in terms of quality preservation of the fresh mushrooms. A polyolefin film with respective gas permeabilities of 166 and 731 mlm-2h-1atm-1 to O_2 and CO_2 established an equilibrated atmosphere of 1.7-2.4% O_2 and 4.1-5.6% CO_2 inside the package at 10 ^{o}C. This polyolefin film was shown to contribute to preserving the freshness of the mushrooms. Temperature fluctuations between 5 and 15 ^{o}C did not induce a harmful atmosphere inside the polyolefin package, though high temperatures accelerated the quality loss (Kang *et al*.2001).

Jasinghe *et al*. (2006) investigated that ultraviolet (UV) radiation-induced conversion of ergosterol to vitamin D2 in fresh mushroom of enokitake mushrooms. A variety of chromatographic techniques were used to study the chemical properties and composition of total lipids in fresh and freeze-dried samples of the edible fungus *F. velutipes* (known as enokitake in Japan). The total lipid content (average of 5 samples) was 0.43% FW (3.58% DM) in fresh samples, and 3.43% FW (3.53% DM) in freeze-dried samples. In fresh samples, the phospholipid and neutral lipid fractions each constituted over 40% of the total lipid content while glycolipids accounted for 14.8%. The predominant lipids in these fractions were triacylglycerol (64.4% of the neutral lipid fraction), cerebroside and acylsterylglucoside (35.8 and 28.5%, respectively, of the glycolipid fraction), and phosphatidyl choline and phosphatidyl ethanolamine (48.0 and 42.2%, respectively, of the phospholipid fraction). Freeze drying only affected these values slightly (Takenaga et al.,1995).

Insect-Pests and Diseases

Various competitors, diseases and insect-pests assocated with mushrooms are described below.

Moulds: Green mould is the most important competitor associated with winter mushroom. Addition of wood (*Quercus acutissima*) vinegar to the sawdust and potato dextrose agar media used for the cultivation of *F. velutipes* @ 10% not only completely inhibited mycelial growth of *Trichoderma harzianum* but also promote the growth *F.velutipes* (Chang *et al*., 1995) . Jiang (2001) studied the the effect of 5 fungicides on

the hyphal growth of *F. velutipes* and contaminated moulds. Chlorothalonil and iprodione are reported to be most effective in managing the moulds associated with winter mushroom. Both of their average inhibitory rates were higher than 90%. Copper hydroxide had the least effect, with an average inhibitory rate lower than 5% in 0.667-0.883 g/litre. Copper hydroxide and chlorothalonil exhibited the strongest inhibition on contaminated mould. The suitable concentration of copper hydroxide was from 0.7-0.8 g/litre. Ogawa *et al*. (1975) reported that it is necessary to add benomyl to the medium before sterilization, @ *F.velutipes* 40-60 ppm to avoid contamination.

Bacterial rot: *Pseudomonas tolaasii* is the main bacterium associated with *F.velutipes*. It results into brown or black lesions on the fruit bodies(Shirata *et al*.,1995; Suyama and Fujii, 1993). The bacterium produced 8 toxins, tolaasin I, the main which plays an important role in the development of symptoms on fruit bodies Shirata (1996).

Soft rot : *P. agaricicola* causes soft rot and produced pitting on the fruit bodies of winter mushroom. SEM studies demonstrated the sequential removal of hyphal wall layers, thereby exposing the chitin skeletal matrix, which was then degraded. A second type of damage typified by collapsed, shrivelled, and in some cases lysed hyphal cells was also observed. Culture plate assays revealed that *P. agaricicola* produced chitinase. This, together with earlier evidence of a β -glucanase enzyme, accounted for the degradative ability of the pathogen (Gill and Tsuneda,1997). Okamoto *et al.* (1999) identified the bacterium as *P. agaricicola* sub sp. *carotovora* causing soft rot of winter mushroom.

Virus-like particles: Presence of virus-like particles of ca. 50 nm diameter associated with the dsRNAs confirmed by electron microscopic ohas been also reported in winter mushroom (Magae and Hayashi,1999).

Nematodes: Nematodes like *Aphelenchoides composticola, Ditylenchus myceliophagus*, *Paraphelenchus myceliophthorus* and *Rhabditis* species are associated with this mushroom (Fang and Luo,1992; Tan *et al*.,1992). *Tylopharynx foetidus* exhibited strong pathogenicity to the mycelia and fruit body of *F. velutipes* and also found to be associated with soft rot disease caused by *Cladobotryum varium* of mushrooms (Cai *et al*,1999).

Mites: The pygmephorid *Luciaphorus auriculariae*, is regarded as a destructive of *F. velutipes* (Zou *et al*. 1993).

Okabe (1993) reared individuals of *Histiogaster* sp. separately on colonies of *F. velutipes* and development period, fertility and the longevity of females were observed at various temperatures. The mites developed faster when they were reared at temperatures of 20, 25 or 30 ^{o}C.

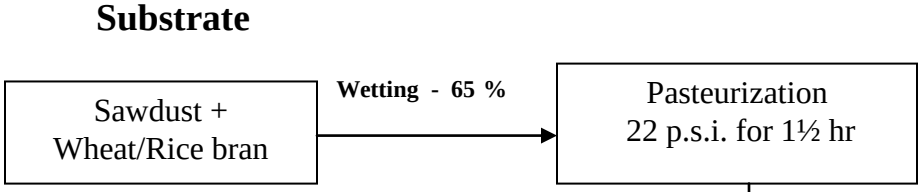
Flies: *Lycoriella* spp are associated with winter mushroom. A temperature of 20 ^{o}C is the most congenial for the development of these flies and they lay eggs which are oval 0.17 mm in diameter and 0.27 mm in length. The average egg period is 4 days. The body length of each instar from 1st to 3rd was 0.7, 1.5 and 4.5 mm, respectively (Choi *et al.*, ,1997).

Spent mushroom substrate management

Substrates used for the cultivation of winter mushroom can be used in the production of vegetable crops like tomato in hydroponics (Lee *et al*., 2005).

. Marketable yields of tomato plants grown in 4 l of RSE on non-recycled hydroponics was 6% higher than those in perlite medium on recycled hydroponics. Total porosity and container capacity of RSE were higher. The amounts of P, K, Mg and Ca in RSE after tomato cultivation also increased.

Flow Chart of *Flammulina* production



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