STUDIES ON THE FUNGUS *FLAMMULINA VELUTIPES*

by

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GOLDEN MUSHROOMS (*F. VELUTIPES*) GROWING ON PULP WASTES.

ABSTRACT

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Key Words : *Flammulina velutipes*, mushrooms, pulp wastes, sawdust, nucleus, meiosis, mycelium, cytology.

Local ligninocellulosic waste materials were examined as substrates for growing *Flammulina velutipes* mushrooms. Mushroom production was favoured by substrate moisture content in the range of 125-150%, (substrate supplementation with wheat bran) and a vegetative run temperature of 25°C for 2-3 weeks. Cropping time was 34-49 days and Biological Efficiency (B.E.) with wheat bran supplemented aspen sawdust and pulp waste mixtures was close to 100 percent. There was significant increase in yield when the amount of wheat bran in the substrate was doubled. Spruce sawdust gave significantly lower yields than aspen sawdust and yield with pulp waste equaled the best yields obtained with aspen sawdust. Bark and inner bark fibres delayed cropping time as compared to other substrates.

Cytological examination revealed irregularities in the nuclear cycle. The oidia were uninucleate, freshly shed basidiospores were binucleate, clamped mycelia were mostly dikaryotic and some cells were multinucleate, and simple septate mycelia were usually multinucleate, trama and basidia were binucleate and stipe hyphae were multinucleate (3-12 nuclei per hyphal cell). Nuclei stained with 4', 6 diamidino'-2-phenylindole (DAPI), a DNA specific dye, fluoresced pale blue. Some cells contained fluorescing yellow particles in the cytoplasm. During meiosis, spindle orientation was hemichiastobasidial. A post-meiotic mitotic division in the basidiospores resulted in binucleate basidiospores. Examination of the hymenial layer of the gill with SEM revealed a variation in basidiospore numbers and size.

Preliminary elemental analyses of pulp wastes and of mushrooms growing on the pulp wastes indicated that lower than expected levels of heavy metals were present in both the materials.

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TO MY PARENTS

NIRANJAN & GURBANS

GENERAL INTRODUCTION

This study was aimed primarily at growing *Flammulina velutipes* (Curt.: Fr.) Sing., mushrooms on local pulp and paper mill wastes, wood chips of aspen (*Populus tremuloides* Michx.) and black spruce (*Picea mariana* (Mill) B.S.P.) in Thunder Bay, Canada. Currently, ligninocellulosic pulp wastes are largely being disposed of by incineration or by introduction into landfills and there is a need to find better uses for these materials. Because *F. velutipes* produces edible mushrooms and grows on wood, it was reasonable to expect it to fruit on pulp wastes. Because of potential pollution problems with pulp wastes, comparisons of mushroom yield were also made of aspen and black spruce. In growing mushrooms on these substrates, culture conditions reported to be optimal in the literature were followed (Tonomura, 1978; Chu-chou, 1983; Kinugawa and Furukawa, 1965).

The second objective of the work was to cytologically examine the hyphae of *F. velutipes.* There have been no recent cytological studies dealing with nuclear division in the basidium nor vegetative mycelium of the species. In view of abnormalities in the nuclear cycle of some fungi (Olive, 1953; Setliff, 1970; Boidin, 1971; McLaughlin, 1982), the nuclear life history of *F. velutipes* was re-examined for deviations from the normal dikaryotic pattern, which may account for the reported genetical and physiological variations among strains of the species (Aschan, 1953; Takemaru, 1961).

The thesis reviews mushroom cultivation, wastes as mushroom substrates, and cytology of *Flammulina velutipes.*

MUSHROOM CULTIVATION

Mushrooms have been the subject of folklore, mystery, and fascination since ancient times. Buller (1915) noted that Greeks and Romans were familiar with many kinds of edible and poisonous fungi. Romans in particular had developed elaborate recipes for cooking the most desirable species. However, the oriental people were ahead in mushroom growing. The padi - straw mushroom [*Volvariella volvacea* (Buller: Fr.)Sing.] was being cultivated 3,000 years ago on rice straw in Southeast Asia (Forsyth,1968). Japanese historical documents referred to the edibility of the shiitake mushroom (*Lentinus edodes* [Berk.] Sing.) as early as 199 A.D. (Singer, 1962). Cultivation of mushrooms in the western hemisphere first began in France with *Agaricus* species. Buller (1915) reported that the first scientific description of the process was given by Tournefort in 1701. By 1800 underground caves of Paris were widely used for mushroom growing. Mushrooms were introduced to the New World around 1885 and were grown in special buildings by 1894 (Snetsinger, 1970). The process essentially involved composting horse manure that was then seeded with mycelium or spawn. Spawn was prepared by growing mycelia on washed horse manure in bottles. This technique was later replaced by cereal grains, bran mixtures, and combinations of peat with organic refuse (Lambert,1938). In the 1930's, replacement of horses by cars meant a decrease in the availability of horse manure for the mushroom industry. In 1938, Dr. Sniden presented a formula for making synthetic compost having straw, urea, and wheat (Snetsinger, 1970). Later, other materials like sawdust were used in mushroom production (Block *et al.*, 1958; Tonomura, 1978).

At present, the wood decomposing fungus, *F. velutipes* is commercially being produced in Japan [90 % of total output], and in Taiwan [10% of total output] (Chang and Hayes, 1978). Several other countries are actively involved in the research of a wide variety of edible mushrooms belonging to different mushroom species with different tastes and textures. In oriental countries, extensive industries have developed around the cultivation of mushrooms. Japan's major agricultural export is Shiitake (*L.*

edodes) which is cultivated on hardwood logs (Leatham, 1982). In China, India, and Japan *V. volvacea* , *Pleurotus ostreatus* (Fr.) Kummer, and *Pleurotus sajor-caju* (Fr.) Sing. form the basis for a profitable industry (Baker,1934; Leatham,1982; Forsyth, 1968). In Taiwan, *Pleurotus cystidiosus* Miller is commonly cultivated (Jong and Peng, 1975). In North America, the species that is most commonly cultivated and consumed is the button mushroom, *Agaricus brunnescens* [= *A. bisporus* (Lang.)Imbach] and this industry is only beginning to show signs of diversification (Farr, 1983). Increasingly, however, there are a number of mushroom species that are being cultivated by small entrepreneurs in North America. Shiitake and oyster mushroom are the two notable examples that have been recently commercially grown in this part of the world (Leatham, 1982). In Canada, edible mushrooms of *Agaricus* spp. were grown under greenhouse benches as early as 1912. In 1985, Canada produced 48.5 million kg of edible mushrooms with a farmgate value of $132.7 million. In the same year mushrooms accounted for 19% of farm value of commercial vegetable crops in Ontario. Ontario was leading all other provinces in mushroom production. Canadians consume about 5% of the world's production of mushrooms. Canada's average rate of increase in mushroom consumption per person per year in 1985 was about 84 g and ranked second to the 134 g consumed in West Germany. In 1984, Canada imported 24.5 million kg of mushrooms (Rinker, 1986).

Nutritionally, mushrooms are a source of protein, B-vitamin, and various minerals (Block *et al.*, 1958). On a dry weight basis, cultivated mushrooms ranked above most common vegetables in having a protein content of 26.9% as compared to 22.5 % in lima beans, 21.6% in green beans, and 26.1% in green peas (Wooster, 1954). Humans can digest 72-83% of the nitrogen in mushrooms (Wooster, 1954; Chang and Hayes, 1978). In addition,the mushrooms are a source of several vitamins including thiamine, riboflavin, niacin, biotin, and ascorbic-acid; the nutritional value is, however, species specific (Chang and Hayes, 1978).

WASTES AS MUSHROOM SUBSTRATE

Because edible mushrooms serve as good sources of food, and at the same time are nature's most active decomposing agents of ligninocellulosic material, use of waste material in their cultivation provides a method of upgrading the wastes into a valuable material. Large amounts of solid pulp wastes are generated from the pulping process. Another industrial byproduct with little use is sawdust. About 40% (i.e. 10 million m^3) of wood waste generated from wood processing industries is dumped in Canada (Manning, 1972). In 1986, 860 494 m^3 of pulp and wood residues were wasted in Ontario (Statistics Canada,1986). These waste materials are readily available, plentiful, and inexpensive in Thunder Bay.

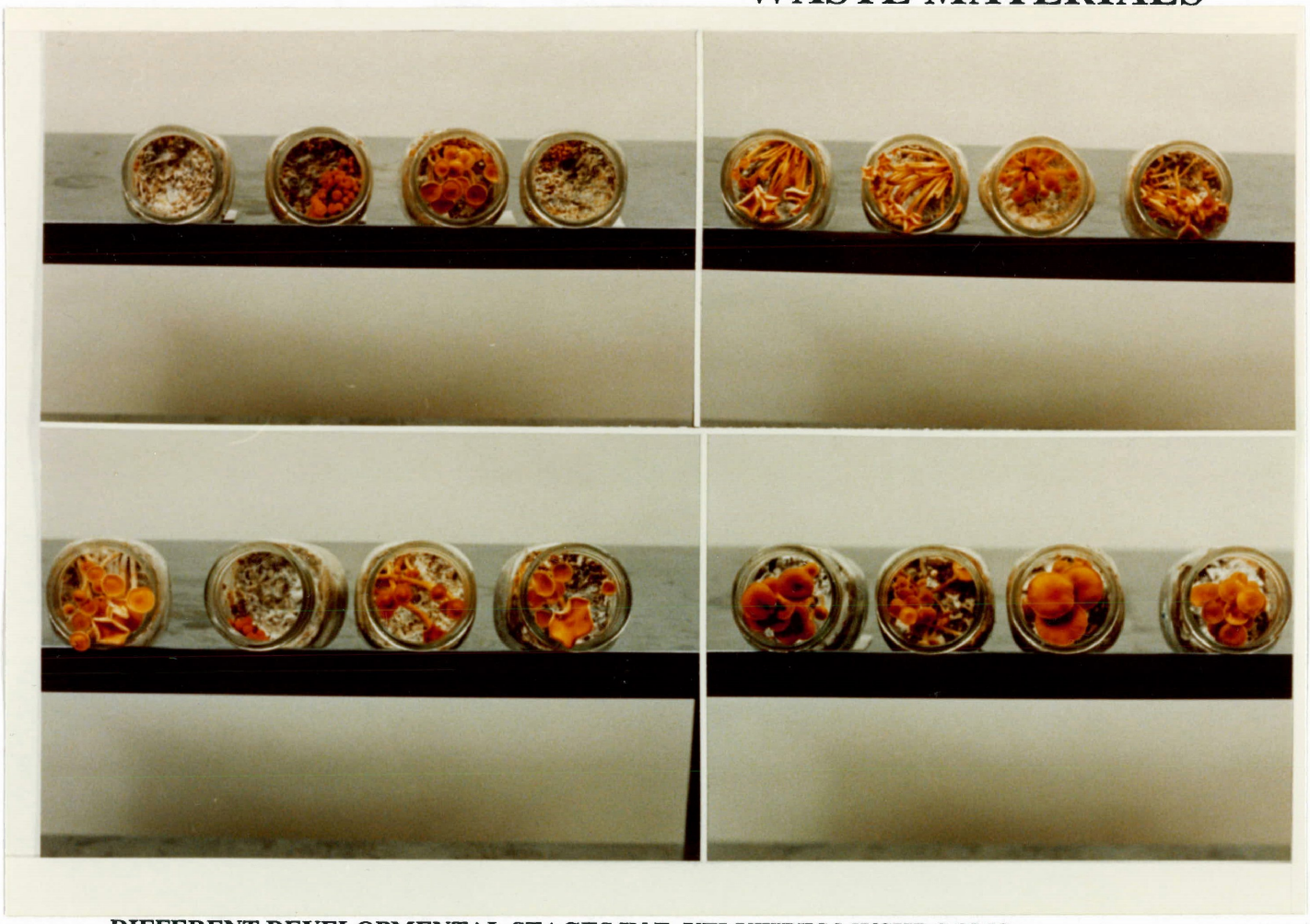
Wood decaying fungi like *F. velutipes* are primary decomposers of the three most abundant organic compounds i.e. cellulose, hemicellulose, and lignin. They have the capacity of growing on high carbon:nitrogen ratio materials and producing fructifications (Kirk and Highley, 1973). This fact, coupled with the availability of tremendous amounts of wastes in Ontario, provides a means of better utilization of wood wastes from pulp mills and sawdust of underutilized hardwood species such as aspen and softwood species such as black spruce. The latter species contributes 90% of the wood wastes in the province. Growing *F. velutipes* has some advantages over other commercial species. Unlike *Agaricus* species, it grows directly on non-composted substrates, and as composting is not required, there is a saving in terms of cost and time. The bulk of the crop is obtained in the first flush of mushrooms and the turnover rate per year is still higher than *Agaricus* species if one foregoes second or third flush. In comparison to shiitake (*L. edodes*), its cultivation cycle is shorter. The former species requires two to eight months to crop after inoculation (Leatham, 1982). *F. velutipes* required 34-49 days to crop after inoculation in present study. Finally, there are no health risks involved in cultivation of this species as compared to those of *P. ostreatus*. Spores of the latter species cause allergic reactions similar to hay fever in most people (Muller

and Gawley, 1983), and in China, high incidence (91.8%) of allergy caused by inhaling spores was reported (Yin *et al.*, 1983).

CYTOLOGY OF *FLAMMULINA VELUTIPES*

Genetic manipulation plays an important role in food production from animals and plants and there is no reason to assume this science cannot be applied to improving mushroom yield. However, very little work has been conducted in this area on the fungi in general and *F. velutipes* in particular. The genetics and sexuality of this species have been subjected to periodic study and morphological as well as biochemical mutants have been described in the literature (Aschan, 1953 and 1960; Takemaru, 1961). For instance, variations exist among strains in the formation capacity of some enzymes and in the activity of these enzymes, it is not clear whether these enzyme systems are influenced by nuclear genes or by the cytoplasm (Aschan,1953). In view of these variations, differences between mycelia resulting from basidiospores of the same basidiocarp are natural, considering recombination possibilities implied by meiosis (Aschan, 1953; Takemaru, 1961). However, there were few cytological studies of *F. velutipes*. In this study, the nuclear life history of the fungus was examined from spore to basidium. Variations in the number of basidiospores on the basidia has been documented in several species of *Agaricus* (Kerrigan and Ross, 1987) and in *Coprinus cinereus* (Schaff.: Fr.) S.F. Gray (McLaughlin, 1982). *F. velutipes* was described as being tetrasporic in the literature (Aschan, 1952 and 1954; Brodie, 1936; Buller, 1958; Takemaru, 1961) and so the hymenium of *F. velutipes* was examined with the scanning electron microscope to confirm these observations.

CULTIVATION OF *FLAMMULINA VELUTIPES* MUSHROOMS ON LIGNINOCELLULOSIC WASTE MATERIALS



DIFFERENT DEVELOPMENTAL STAGES IN F. VELUTIPES MUSHROOMS.

LITERATURE REVIEW

INTRODUCTION TO *FLAMMULINA VELUTIPES*

Flammulina velutipes is a ligninocolous fungus that produces edible mushrooms. The fungus is cosmopolitan in distribution (Tonomura, 1981) and its fruit-bodies are found on tree stumps and logs of elm, willow, aspen, beech, basswood, and sugarmaple (Buller, 1931) throughout the year. Because of its ability to endure much lower temperatures than other agarics, it has also been referred to as a "winter" mushroom (Stewart, 1918). Other common names are the "golden" mushroom, the "velvet-stemmed" mushroom, and in Japan it is known as "enokitake" (Chu-chou, 1983; Tonomura, 1978).

The fruit-body has a velvety stipe which is bright cinnamon in color, darker at the base, stuffed to hollow, and usually thick and tough. Lammellae are sinute, adnexed, rather broad, creamy to yellowish in color (Ainsworth and Sussman, 1973). They are wedge shaped and their structure is Aequi-hymeniiferous, i.e., the hymenium develops in an equal manner over the gill surface. Hymenium development is hemiangiocarpic, i.e., the hymenium is formed on the inside of the primordium from the earliest stages of development and becomes exposed at maturity (Singer,1967). The hymenium has cystidia on the sides of lamellae that are conic, rather acute and 8-12 um broad (Buller,1931; Singer, 1967). The pileus is glabrous with dermatocystidia (cystidioid bodies of the cortical layer). The spore print is white and the spores are hyaline, smooth, long, elliptic, 7-8 x 5um, and obliquely apiculate. Spore development is a rapid process taking about 47 minutes from appearance of four rudiments of spores to spore discharge. The fungus is capable of shedding spores at 1^{o} C (Buller, 1958). In this connection, Stewart (1918) noted "... it is the only wild mushroom obtainable in quantity during [the spells of mild weather] in the

winter (New York). It may be frozen solid for days without affecting its quality or its ability to resume growth upon the return of mild weather... its flavour is excellent. Most mushroom eaters of the writer's acquaintance pronounce it first class ... another desirable quality is the long time it may be kept ...."

Taxonomically, *Flammulina* (Curt.: Fr.)Sing. (*Collybia*, Kummer; *Pleurotus*, Qúel. 1886; *Gymnopus*, Murr.; *Myxocollybia*, Sing.) belong to Subtribe Flammulinae, Tribe Marasmieae, Family Tricholomataceae, and Order Agaricales. The Genus *Flammulina* occupies a somewhat isolated position and only one species, *F. velutipes* occurs in the genus (Singer, 1967). Nuclear condition of the basidiospores is an important taxonomic criterion and its nearest relatives in Tribe Marasmieae have uninucleate spores. However, basidiospores of *Flammulina* have been described as binucleate by some (Duncan and Galbraith, 1972) and uninucleate by other studies (Raper, 1978). If, as Singer (1967) has suggested, this feature is of taxonomic importance, then cytological examination of basidiospores is important.

A brief literature review on the genetics of this species was made essential before attempting a cytological study. The mating system has been reported as being tetrapolar or bifactorial, i.e. sexuality is controlled by two factors, "A" and "B" with functional differences (Aschan, 1954; Takemaru, 1961). Different "A" factors permitted clamp formation and different "B" factors allowed the nuclei to migrate. In a compatible mating, where "A" and "B" factors were different, clamp bearing hyphae were found throughout both participating mycelia, i.e. complete dikaryotization occurred. In common "B" mating, where "A" factors were different but not the "B", clamp formation was restricted to the contact zone, i.e. limited dikaryotization occurred. The "A" and "B" factors, each with a complex of loci, functioned as a single unit physiologically (Aschan, 1954; Takemaru, 1961).

F. velutipes is white rot fungus that is capable of decomposing all important structural components of wood, *viz.* holocellulose and lignin (Tonomura, 1978). Carbohydrates in the wood tissue are destroyed by the hydrolysis reaction with the help of extracellular cellulase enzymes produced by the fungus. Lignin decomposition apparently occurs by an oxidative process and its removal depends strongly on the type of wood being decayed. Among various nitrogen sources, malt extract was generally stimulatory toward fungal lignin depletion (Kirk and Highley, 1973). Buller (1931) commented on the preference shown by *F. velutipes* for the wood of newly killed trees and its semiparasitic habit (i.e. it is frequently found on the trunks of living trees, and often has been suspected of being pathogenic under certain conditions). Griffith and Barnett (1967) reported *F. velutipes* to be necrotrophic or a destructive type of mycoparasite that had the ability to parasitize and destroy spores and vegetative cells of host fungi such as *Ceratocystis fimbriata* Ellis & Halst. and *C. fagacearum* (Bertz) Hunt. Its ability to decompose and fruit on wood would facilitate its growth on ligninocellulosic wastes and in this regard a brief review of its physiology is now given.

PHYSIOLOGY OF *F. VELUTIPES*

A number of studies in the past have focused on the physiological parameters related to fruit-body and mycelium growth on synthetic media. Optimum temperature for mycelial growth was about 25^{o} C (Brodie, 1936; Takemaru, 1954; Kinugawa and Furukawa,1965). Aschan (1958) reported that optimum shifted from 25^{o} to 20^{o} C with increasing length of growth period. Thiamine was important for vegetative growth and addition of yeast extract to cultures resulted in increased mycelial growth but inhibited fruiting (Plunkett, 1953). The production of well developed fruit-bodies had an upper temperature limit close to 20^{o} C (Plunkett, 1953; Aschan, 1954 and 1958). Kinugawa and Furukawa (1965) examined a range of temperatures from 5^{o} C to 25^{o} C. They reported 15^{o} C as being optimum for fruiting and at

25° C fruit-body formation was suppressed. Mycelial growth was inhibited by nitrogen salts and urea (Gruen and Wu, 1972) and acenaphthene (a hydrocarbon isolated from coal tar that induces polyploidy in plants) caused 66% inhibition in mycelial growth (Tews, 1968). Addition of fructose 1,6 diphosphate at 0.0065% increased growth of monokaryotic mycelium, but at higher concentrations response varied among strains (Aschan 1956 and 1960a). Ammonia compounds and amino acids were readily utilized as nitrogen sources; inorganic nutrients like Mg^{2+} and PO_4^{3-}, and trace elements such as Fe, Zn, Mn, Cu, Co, Mo, and Ca were also required by both mycelium and fruit-bodies (Tonomura, 1978). During the process of fruit-body maturation, rapid elongation began at 2 cm length and continued for 2-3 days until the length was about 7 cm. The final length averaged 8 cm, but some fruit-bodies reached 12-14 cm in length. The pileus could attain a mean diameter of 1.5 cm at this stage (Gruen and Wong, 1982). Gruen (1969) reported that growth factor(s) which were necessary for stipe elongation were produced by lamellae in the cap and production of this diffusate was stimulated by the addition of glucose, although the nature of this diffusate was unknown. Further work established that stipes did not depend on the diffusate during almost half of the total growth period (Gruen, 1976). Elongation phase of the stipe alone was controlled by the diffusate and its release stopped at the end of this phase (Gruen and Wong,1982). The nature of the stimulate and why lamellae stop releasing this diffusate is unanswered at present. There was an increase in dry weight of the fruit-bodies during elongation (Plunkett, 1953) and this increase was mainly due to stipe weight (Gruen and Wu, 1972). Nutrients and water were supplied by the vegetative mycelium during most of the growth period and as the growth rate declined, the fruit-bodies became more independent of the mycelium (Gruen and Wu, 1972), accompanied by a decrease in concentration of the nitrogen supplied by the mycelium to older fruit-bodies; this may have been due to diminished translocation of nitrogen and also due to loss of nitrogen through spore discharge (Gruen and Wong, 1982).

In a cluster of fruit-bodies, the larger ones obtained nutrition partly from carbohydrates in the medium, partly from cellular constituents stored in the mycelium, and partly from smaller fruit-bodies (Kitamoto and Gruen, 1976). Fruit-bodies in a group inhibited each other by competing for materials supplied in a limited amounts from the mycelium and distributed among individuals proportionally to ranks; therefore size of the fruit-bodies was influenced by the number of fruit-bodies allowed to elongate on the mycelium (Gruen,1979). In larger fruit-bodies, the main low molecular weight storage carbohydrates were trehalose, arabitol, and small amounts of mannitol and in the mycelium and small fruit-bodies, glycogen was the main storage carbohydrate. Glycogen was broken down in both the mycelium and small fruit-bodies during the growth of large fruit-bodies (Kitamoto and Gruen, 1976). In higher fungi, external as well as internal factors that regulate the fruiting process are largely unknown, and a large fraction of all species have never fruited in the laboratory (Raper, 1966). For *F. velutipes*, Plunkett (1953) demonstrated the critical importance of environmental conditions upon fruiting with the use of synthetic media. It was found that primordia and stipes of the fruit-bodies were formed in darkness, but there was no expansion of caps unless light was provided. Growth of the pileus was greatly stimulated even by weak light and only the blue end of the spectrum activated normal pileus formation; wavelengths longer than 470nm were ineffective in causing pileus formation (Plunkett, 1953; Aschan, 1960). Presence of light and low carbon-dioxide concentration were essential for normal fruit-body development (Plunkett,1953; Aschan,1954; Takemaru, 1954; Tonomura,1978). There was significant increase in fruiting by increasing the depth of the medium (Plunkett, 1953). Initial pH of 5.7 of the medium was reported to be favourable for fruiting and varying conditions for growing inoculum had no effect on fruiting (Aschan, 1958). Increased glucose concentration delayed fruiting (Plunkett,1953; Aschan, 1958). They explained that perhaps this delay was because the fungus had to dilute the glucose medium to a level favourable for reproduction. Time required to produce fruit-bodies was reported to vary with the different strains (Aschan and Norkans, 1953). For commercial production of the species in Japan, at first wood logs were utilized in

its cultivation, but the mushrooms produced were unsuitable for commercial purposes (Tonomura, 1978). Shiio *et al.* (1974) described a vat type of cultivation method with a shorter cropping time, but contamination was reported even with low nutrient medium. Less contamination was reportedly observed when pieces of fruit-bodies were spread over the surface of the medium. At present, bottle cultivation is preferred in the commercial process (Shiio *et al.*, 1974). Tonomura (1978) gave a detailed account of cultivation process being followed in Japan. Four parts of native hardwood species sawdust are mixed with one part of rice bran, filled in polypropylene bottles, and sterilized. They are next inoculated with sawdust spawn and placed in a culture room at 18^{o}-20^{o} C. After 20-25 days, bottle caps are pulled off, inoculated spawn is removed and the surface of the media is made smooth for fruiting. The bottles are then placed in the dark at 10^{o}-12^{o} C, humidity at 80-85%. Fruiting is encouraged by adjusting the humidity levels. After 5-7 days, when the stipe is 2 cm long, the mushrooms are exposed to 5^{o}-8^{o} C, humidity at 75-80%. Fruit-bodies are harvested when they reach 13-14 cm in length. This process takes about 50-60 days. Quantity as well as quality declines with subsequent cropping. After harvesting, 47-64% Biological Efficiency was reported. Sawdust from trees native to New Zealand was experimentally examined as substrates for cultivating this species (Chu-chou, 1983). For some other mushroom species in addition to sawdust, a range of wastes (depending on their availability in that particular region) were examined as potential substrates for mushroom production. These are discussed below.

WASTES AS SUBSTRATES IN MUSHROOM CULTIVATION

In Japan, calcium salt extracts of sulphonated polysaccharides, ligninocellulose and milled wood lignin were utilized to accelerate mycelial growth and fruit-body formation in *P. ostreatus* and *F. velutipes*. In both species, addition of 1% of this accelerator gave positive results. At the 2% level, abnormal fruit-bodies were formed with *P. ostreatus* whereas normal fruit-bodies resulted with *F. velutipes*.

Yield was 1.2-1.3 times greater with the latter species (Inaba *et al.*, 1984). Mushrooms of *P. ostreatus* were obtained from rice straw, rice hulls, newspaper, and pine sawdust supplemented with rice bran (Hashimoto and Takahashi, 1974) and on leaves (Idei and Yoshizawa, 1984). In India, the same species was cultivated on waste paper and tea leaves supplemented with wood ash and yeast (Harsh *et al.*, 1981); *Pleurotus sajor-caju* was cultivated on banana pseudostems and chopped paddy straw (Jandaik and Kapoor, 1974), on waste paper, rice straw, sugarcane bagasse, coir waste, wood shavings, *Delonix* flowers, and *ragi* ears (Sivaprakasam and Kandaswamy, 1981), on yeast mud, ground nut cake, cotton seed powder and on horsegram powder (*Delichos biflorus*) [Bano and Rajarathnam, 1982]. In the Phillipines, abundant fruit-bodies of *Auricularia polytricha* (Mont.) Sacc. resulted from a 5: 2: 1: 1.5 ratio of sawdust, rice straw, rice bran, and ipil-ipil leaves (Giron and Ballon, 1981). In British Columbia, thermomechanical and kraft pulp mill wastes enriched with soya flour and apple pomace were examined as substrates for *P. ostreatus* and *P. sajor - caju* (Mueller and Gawley, 1983). Obviously wood-inhabiting mushrooms can grow on wood residues and waste materials; however production of *F. velutipes* mushrooms on pulp waste materials, aspen wood and black spruce remain untested in Canada. A brief review of literature on wood waste production in Canada follows.

WOOD WASTES

In Canada, one third of the land area is covered by forests (Filion and Polis, 1975) and 146 pulp and paper mills represent the largest industries (Manning, 1972; Bruely,1974). The bulk of residue generated by these industrial sources is largely wasted at present (Table 1). In a survey of residue from the pulp and paper mills in Toronto (1984), it was found that of 164 000 dry tonnes residue produced annually, 45 000 dry tonnes were being dumped! There is a need for more research that will lead to greater utilization of this resource. In the present work, sawdust of trembling aspen (*Populus tremuloides*), sawdust of black

spruce (*Picea mariana*) and solid pulp wastes were examined as substrates for the production of *F. velutipes* mushrooms. Wheat bran was used as the main nutrient supplement. A brief review of these materials follows.

SUBSTRATES UTILIZED IN PRESENT WORK

Trembling aspen

Aspen's range is transcontinental through Canada. It is one of the most widely distributed species in North America (Lamb, 1967). Although abundant, aspen is often viewed as a commercially underutilized species in Canada (Eugene, and Michael, 1985). Some of its present uses are in paneling doors, fabrication of boxes and crating, for concealed parts and core stock in furniture, in writing, tissue paper etc. Aspen is fast growing, short lived (60-80 years), and usually of small size and average height, 15-18m and diameter 20-40 cm. Aspen sapwood is whitish to creamy colored and the heartwood is usually stained and decayed. The wood is highly susceptible to heart rot and the presence of unsound knots (as well as tension wood) makes its use as a lumber species very low (Eugene and Michael,1985; Lamb, 1967). In view of aspen's underutilization and occurrence of *F.velutipes* fruit-bodies on hardwoods in nature, sawdust of aspen was chosen as one of the materials to be tested for fruit-body production in the laboratory.

Black spruce

Spruces are the dominant tree species in Canada and contribute 40% of the coniferous volume and one-third of the total volume of all Canadian species (Hearnden, 1975). Black spruce (*Picea mariana* [Mill.] B.S.P.) is one of the most widely distributed tree species in Canada and ranges from Newfoundland to Alaska. Black spruce supplies 90% of the pulpwood to the 38 pulp and paper mills in Ontario (Hearnden, 1975; Rogers, 1961). Sawdust of black spruce is used in pulping, but the quality of

Table 1. Mill and forest residue in Canada.

	Estimated oven-dry tonnes x 10^6		
Source	Amount available	Amount used	unutilized
Mill residues	16	7	9
Logging residuals, unmerchantable trees, bypassed stands, etc.	102		102
Potential unharvested material on productive land	108	–	108
Total			219

Source : Clarke and Sastry, 1982.

pulp is inferior to that obtained from wood chips (Bruely, 1974). Some other uses are as particle board, as mulching material, as ground covers, and in landfills (Philip and Schroeder, 1975; Philip and Azarniouca, 1981).

Utilization of aspen and spruce sawdust in the present study permitted comparison between hardwoods and softwoods. There is little difference in their elemental composition. Hardwoods are reported to have 49.01% carbon, 6.12% hydrogen, 44.07% oxygen, and 0.10% nitrogen. Softwoods have 50.34% carbon, 6.06 % hydrogen, 43.24 % oxygen, and 0.05% nitrogen. In terms of chemical characteristics of aspen and spruce, aspen has 16.3% lignin, 80.1% holocellulose, 3.4% acetyl and 0.4% ash. Spruce has 28% lignin, 70.5% holocellulose, 0.4% acetyl and 0.3% ash (Browning, 1963).

Solid pulp wastes

Pulp wastes have served as a food source for fungal growth (Pilon *et al.*, 1982). These ligninocellulosic wastes have a high ash content. A typical 1 000 tonne/day pulp mill produces 5-10 tonnes of solid wastes per day. Northwest Ontario mills produce about 200 tonnes/day of solid pulp waste [Kapoor (pers. comm., 17 March 87)]. This waste at present is disposed of by incineration or is introduced into landfills. Heat value of these wastes is low, approximately 0.24 Kcal/kg., and landfill sites are becoming increasingly limited. The wastes were utilized as fertilizers and a number of studies described below have dealt with the effects of solid sewage wastes as fertilizers and problems associated with their usage as soil amendments in crops and forestry plantations.

Increase in the yield of cereals was reported as a result of sewage waste fertilization. This increase was reported for cereals such as corn and rye (Cunningham *et al.*, 1975), for wheat (Sabey *et al.*, 1977), for sorghum (Mays *et al.*, 1973), and for barley (Vlamis *et al.*, 1978). Some other experimental

applications were conversion to edible protein for animal and human consumption (Turnbull, 1982) and fertilization of forestry plantations (Brockway, 1983). There has been a certain amount of concern regarding the presence of heavy metals, especially cadmium in these wastes. Cadmium is in the same group as zinc in the periodic chart and the ubiquitous association of these two elements in nature has been reported (Morrison, 1979). Wastes containing low Zn levels with respect to Cd may cause cadmium buildup in soil and vegetation. There is no legislation controlling the amount of Cd present in the food (Thomas *et al.*, 1972). For wastes, Brockway (1983) reported Cd level of $\leq$1 mg/kg as non-hazardous and an acceptable ratio of Zn : Cd as $\geq$100:1. For pulp mill wastes this ratio was 108:1 and for sewage waste it was 4:1 (Brockway, 1983). In some studies it was suggested that modest amounts of solid sewage wastes supplied significant amounts of N, P, and other nutrients and improved vegetation yield; but excessive amounts were detrimental to yield (Dolar *et al.*, 1977; Topper and Sabey, 1986). Other studies based on soils amended with wastes for growing vegetables like carrots, radish, cabbage, green beans, sweet corn, and tomatoes reported that there was no appreciable accumulation of heavy metals, and although the level of heavy metals in the vegetables was higher as compared to controls (i.e. without sewage waste fertilization), it did not exceed hazardous limits (Keefer *et al.*, 1986). Chaney (1973) proposed the following upper limits of metals in wastes for application to agriculture land : Zn, 2000 ppm; Cd, 20 ppm or 1% of Zn, whichever is lower; Ni, 200 ppm; B, 100 ppm; Pb, 1000 ppm; Cr, 1000 ppm; and Hg, 10 ppm. Chaney *et al.* (1976) evaluated the long term effects of heavy metals on a number of farms that utilized sewage fertilization for several years. They reached the conclusion that Cu, Ni, Zn, and Cd availability for crop uptake was dependent largely on soil pH and the use of low Cd wastes had little influence on crop Cd levels. They found that the nature and rate of waste applications, and the type of soil determined metal availability to plants, and this was not directly influenced by the concentration of trace metals in the wastes (Johns and Van Laerhaven, 1976). Composting tends to stabilize these wastes and the thermophilic temperatures during this process kills the pathogens (Simeoni *et al.*, 1984). Studies on

utilization of composted wastes have reported increased N uptake by cereals, and reduced availability of Cd and Zn to plants as compared to uncomposted wastes (Simeoni *et al.*, 1984; O'Keefer and Meisinger, 1984). In the last decade, changes in industrial waste treatment practices have significantly reduced the concentration of heavy metals in the wastes (Bruely, 1974).

Wheat bran

While wood and pulp wastes provide lignin, cellulose, monosaccharides, and micronutrients, other essential nutrients must be provided by supplements such as wheat bran. Wheat bran was chosen because it is in plentiful supply in Canada and the fact that other cereal brans have made for good nutrient supplements in the mushroom production (Table 2).

Table 2. Wheat bran composition.

Component	%	ppm	ug/g
Dry matter	89.0		
Crude Protein	14.8		
Crude fat	4.0		
Crude fibre	10.0		
Calcium	0.14		
Phosphate	1.17		
Ash	6.4		
Sodium	0.06		
Potasium	1.2		
Magnesium	0.55		
Sulphur	0.22		
Manganese		100	
Iron		170	
Copper		10.3	
Zinc		95	
Selenium		0.5-1	
Vitamin B_1			6
Nicotinic acid			232

Source : Kent, 1966; Pearson,1970.

MATERIALS AND METHODS

The method of cultivating *F. velutipes* mushroom was based on earlier studies on mushroom cultivation (Block *et al.*, 1956 and 1959; Chu-chou, 1983; Kinugawa and Furukawa, 1965; Royse, 1985; Mueller and Gawley, 1983). It was observed during preliminary experiments that non-enriched substrates (i.e. pulp waste, aspen and spruce sawdust) supported mycelial growth, but they were unsuccessful in producing fruit-bodies. Addition of wheat bran to the substrate improved fruiting, but yields were low. Therefore, other experiments were conducted with a variety of nutrient supplements. Contamination by mites and molds was a problem encountered during the early phases of this work. It was thought that this may partly be due to increased temperatures within the cultivation jars favouring the growth of contaminants. In order to overcome this problem and obtain meaningful yields, metallic canning jar type lids were replaced by heat resistant plastic covers. Small pin holes were made in the plastic prior to autoclaving to improve aeration after inoculation. Incubation chambers were sprayed with insecticide spray or left closed for 16-20 hr at 40^{o} C before starting an experiment. It was found that contamination level was drastically reduced if not totally eliminated. To improve yield, a dish full of water was placed in the chamber to increase humidity (the growth chambers had light and temperature control but lacked humidity control). In preliminary experiments involving wheat bran supplemented aspen, spruce, pulp waste, and their mixtures, it was observed that aspen gave good yields and was available in plentiful supply. Therefore, in subsequent experiments, aspen sawdust was used as the substrate in determining optimal conditions for fruit-body formation.

Canning jars of 500 ml. capacity (Domglas, Toronto, Canada) were used as growth containers; sawdust of aspen and spruce, and solid pulp wastes were obtained from Great Lakes Forest Products, Thunder Bay; other wastes obtained were bark and inner bark fibre of unknown origin. Substrates were enriched with wheat bran (Vita health, Winnipeg, Canada) except where other available nutrients, i.e. barley and coomings (a waste byproduct of malt supplied by Canada Malt Co., Thunder Bay, Canada) were tested. Five different isolates of the species were examined for their ability to fruit. They were 1) Malloch 8. 12. 73/8 - isolated from a fruit-body in a cluster on a stump in 1973. 2) DAOM 129030 - isolated in 1960, the substrate was log slash in a wet depression site in Victoria, B.C. 3) DAOM 188690- isolated in 1982, from a live tree of *Acer saccharum.* 4) and 5) ECS-1669 and ECS-1673 - isolated from fruit-body spores in 1985, the substrate was *Ulmus americana* stump at old Fort William, Thunder Bay, Ontario. Of the five isolates tested for their fruiting capacity with wheat bran supplemented substrates (i.e. pulp waste, aspen and spruce sawdust) during preliminary work, DAOM 188690 and ECS-1673 fruited; however the fruit-bodies of the latter were very viscid and in further experimentation DAOM 188690 was used.

In each jar a 4:1 ratio of substrate: nutrient, i.e. 24 g of substrate and 6 g of wheat bran were mixed (except where different proportions amounting to a total of 30 g, i.e. 1:1, 2:1 and 4:1 ratio of substrate: nutrient were tested). In experiments that tested wood substrate mixtures, equal proportions of each substrate (total 24 g) were supplemented with 6 g of wheat bran (i.e. a mixture of aspen: spruce was 12 g each of aspen and spruce and 6 g of wheat bran; mixtures of aspen: spruce: pulp waste was 8 g of each mixed with 6 g of wheat bran). Tap water was added at the rate of 60 ml (200% moisture level) and thoroughly mixed, except where different moisture levels, i.e. 30 ml (100%), 37.5 ml(125%), 45 ml (150%) and 60 ml (200%)were tested. A small hole was made from the top centre to the bottom of the substrate and surface was made smooth by gentle tapping. The jars were then autoclaved at 121^{o} C and 1.2 kg. per cm^{2} pressure for 20 min. (AMSCO 2022, Pennsylvania, U.S.A). After autoclaving, the jars

were cooled to room temperature and substrate inoculated. The inoculum was agar plugs of 7-10 day old mycelium growing on 2% malt agar (Difco Laboratories, Detroit, Michigan, U.S.A). Roughly 1.5 g of mycelial culture was cut into small pieces and mycelial pieces were placed in the central hole and a few pieces were placed on flat surface of the substrate. The jars were then usually transferred to a dark growth chamber at 25^{o} C (Conviron, Winnipeg, Canada). Different incubation temperatures, i.e. 20^{o} C and 25^{o} C were tested. After at least 90% of the substrate in a jar was covered by mycelium (2-3 weeks time, specified in results section for each experiment), the jars were transferred to a growth chamber at 15^{o} C, and with incandescent lights on. This alteration in environmental conditions induced fruiting. After transfer to this chamber, the jars were wrapped in transparent plastic bags with wet paper towels and loosely secured with a tie. Small pin holes were made in the plastic bags to promote aeration. The fruit- bodies were harvested at maturity (indicated by flattening and upcurling of the pileus). Mushroom yield was expressed in terms of Biological Efficiency (B.E.), i.e. percent yield of fresh mushrooms harvested in grams per gram of dry substrate weight of individual mix (Royse, 1985). Dry weights were determined by assessing the moisture content of the final mix on a wet-weight basis and then subtracting the substrate weight from this value. Other observations relating to stipe length, cap diameter, number of fruit-bodies harvested were made. Confidence intervals comparing means of two independent substrates were constructed using a software program (Hypoth-M , 1980) on apple IIc (California, U.S.A.). The boundry values of a confidence interval depended on the sample means, the sample standard deviations, and the sample sizes and true differences in the means was expressed in the form of strictly positive or strictly negetive intervals. The computational formula was- $(x_1 - x_2) - t\sqrt{s_1^2/ n_1 + s_2^2/ n_2} < u_1 - u_2 < (x_1 - x_2) + t\sqrt{s_1^2/ n_1 + s_2^2/ n_2}$, where t has $n_1 + n_2 - 2$ degrees of freedom. In order to observe if the pH of the pulp waste was appropriate for the fruiting range of the species, this value was determined by stirring 10 g of sample and 15 ml of water, allowing it to stand for 15 min and measuring the pH with an electronic pH meter (Orion research, model 601 A, Boston, U.S.A).

For elemental analysis of pulp waste and mushrooms grown on pulp waste supplemented with 20% bran, the total nitrogen content was determined by an elemental analyzer with 0.3% absolute accuracy (Perkin-Elmer, model 240-XA, Hamilton, Ontario). Other elements in the samples were determined by either ashing for 6-8 hr over flame and dissolving the ash in 10% HCl or ashing for 12 hr at 800^{o} C in an Thermolyne 10500 furnace (Sybron, U.S.A.) and heating the ash twice to dryness in 3:1 ratio of HF:HNO_3, simmering with 10 ml conc. HCl, heating with 10 ml double distilled water and finally dissolving in 100 ml double distilled water (Mitchell *et al.*, 1980). However, all the ash did not dissolve and was filtered. Filter paper with residue was weighed and corrections were made in final calculations. Analysis for 28 elements (Table 10) in the sample solution was performed by emmision spectroscopy (Allied ICP unit, Maryland, U.S.A). The detection limits for Cd and Pb were 0.02ppm and 0.2ppm respectively.

RESULTS

Effect of substrates and substrate mixtures on mushroom yield.

Fruit-bodies appeared on all test media and similar mushroom yields were obtained with the exception of significantly lower yields on spruce (Sp) [Table 3]. The B.E. with aspen and pulp waste was 96% and 84% respectively, however the yield with spruce was significantly lower at 21%. Spruce medium gave between 16-26 g less yield as compared to aspen and a mixture of aspen and spruce yielded between 11-21 g more mushrooms as compared to spruce but gave yields equivalent to aspen. Addition of pulp waste to spruce enhanced yield by 11-23 g, although a similar effect was absent with aspen. Mixtures of aspen, spruce and pulp waste performed well and gave 79-91% yield. There was considerable variation in yields among replications within an experiment and between experiments when they were repeated. During preliminary experiments with these substrates, some replications of a medium fruited while others failed to fruit. The B.E. among subsequent flushes varied from 17-89% for the first, 0-15% for the second and 0-2.5% for the third flush. Therefore in this study harvesting was confined to two flushes.

Usually the mushroom caps were slimy and golden yellow in color. The largest fruit-body in the clusters had a central stipe, 11.5-13.6 cm in length and the pileus was circular, flat, upcurled, 2.8-3 cm in diameter. Fruit-bodies appeared first on pulp waste media with or without aspen, however crop maturation (as described in Materials and Methods) occurred at about the same time on all media (Table 3).

Table 3. Cultivation and yield of mushrooms with substrates having 20% wheat bran (w/w) supplementation.

Substrate	Substrate pH (Range)	Replicate (No.)	Time (range) to crop (Days)	Average yield (g)±S.D. (Total of both flushes)	** B.E.(%)
SP[1]	5.56-5.84	6	36-39	5.8±4	21
AS[2]	5.86-6.97	5	35-36	27±3.4	96
			-26<u(SP)-u(AS)<-16*		
SP, AS	5.66-6.43	6	36-37	22.1±3.4	79
(1:1)			-21<u(SP)-u(SP, AS)<-11		
PW[3]	5.98-6.92	6	34-36	23.4 ±5.2	84
			-23<u(SP)-u(PW)<-11		
SP, PW	5.57-6.38	6	36-37	25.6 ±7.7	91
(1:1)			-27<u(SP)-u(SP,PW)<-11		
AS, PW	5.66-6.94	6	35-36	24.4±4	87
(1:1)			-12< u(SP)-u (AS, PW)<-24		
AS,PW,SP	5.69-6.23	5	35-36	24.6 ±2.6	88
(1:1)			-23<u(SP)-u(AS, PW, SP)<-14		

SP[1] = spruce, AS[2] = aspen, PW[3]= pulp waste. Ratios in the parentheses indicate proportions of the substrates in the final mix. Experiment was repeated twice and the average yield of the two were tabulated.

* Confidence intervals (95%) comparing spruce (SP) with other substrates, other comparisons were not significant.

** B.E. = Biological Efficiency (fresh weight of mushrooms/dry weight of the substrate x 100).

Effect of different nutrients on fruiting and mushroom yield.

The nutrient supplements examined were wheat bran, coomings and ground barley grains. Jars with 100% nutrient failed to fruit. On adding aspen sawdust, fruiting was observed with all nutrient supplements except for barley. The mycelial growth was very dense in aspen barley mixture as compared to other nutrient mixes. Wheat bran and coomings gave nearly equal yields, although the fruit-bodies appeared earlier on the former substrate (Table 4). In the next experiment, wood:nutrient ratio was varied to observe its influence on yield.

Comparison of yields with different proportions of substrate and bran.

Aspen sawdust was mixed with bran in different proportions to test the effect of nutrient supplement on mushroom yield. As the wood level increased and nutrient level decreased by nearly half, there was significant decrease in yield. Yield with a 4:1 proportion averaged 1-12 g higher than with a 1:1 ratio of aspen to wheat bran respectively (Table 5). Both 1:1 and 2:1 ratios gave similar yields, although fruiting was earlier with the latter ratio.

Effect of different moisture levels on the mushrom yield

Mushroom yields were similar at all moisture levels tested and the B.E. varied between 62-80% (Table 6). However, fruit-bodies appeared earlier on media with 150% moisture content and were last to appear on media with 200 and 100 % moisture content.

Table 4. Mushroom yield with three different nutrient supplements added to aspen sawdust.

*Nutrient	Replicate (No.)	Time (range) to crop (Days)	Average yield (g) ±S.D. (Total of both flushes)	B.E.(%)
Bran	6	36-37	15 ±2.6	54
Coomings	5	38-42	18 ±4.8	64
Barley	6	** _	_	—

*Aspen sawdust and nutrients in a ratio of 4:1 respectively.

** - no fruiting. No significant difference between average mushroom yield on bran and coomings (95% confidence interval). Experiment was repeated twice and the average yield of the two were tabulated.

Table 5. Final mushroom yields on different proportions of aspen sawdust to bran after two flushes.

Proportion (Aspen:Bran)	Replicate (No.)	Time (range) to crop (Days)	Average yield (g) ±S.D. (Total of both flushes)	B.E. (%)
1:1	6	36-38	25.3±4.6	90
2:1	6	34-36	25±6.7	89
4:1	6	36-38	19.4±2.1	69
			0.8<u(1:1)-u(4:1)<12*	

*Confidence interval (95%) comparing 1:1 and 4:1 proportions, other comparisons were not significant. Experiment was repeated twice and the average yield of the two were tabulated.

Table 6. Mushroom yield at different substrate moisture levels.

% Moisture*	Replicate (No.)	Time (range) to crop (Days)	Average yield (g) ±S.D. (Total of both flushes)	B.E.(%)
100 (30 ml)	6	36-40	18.3 ±3.8	70
125 (38 ml)	6	34-36	18.2 ±1.9	70
150 (45 ml)	6	34-35	21 ±3.8	80
200 (60 ml)	6	35-36	17.3 ±3.1	62

* No significant difference in yield at increasing moisture levels (95% confidence interval). Numbers in parentheses represent the amount of water added to the jars with 30g substrate mixture. Experiment was repeated twice and the average yield of the two were tabulated.

Use of waste bark, fibre and aspen in *F. velutipes* mushroom production.

There was fruiting on bran supplemented fibre although the yield was 5-16 g lower than aspen and fruiting was delayed by 10 days (Table 7). A mixture of both aspen and fibre was 4-20 g lower in yield and fruiting was delayed by 8 days as compared to aspen. However, on addition of bark to the fibre, yield increased between 1-15 g, but fruit-bodies were the last to appear on this media. Bark and fibre mixture was better than a mixture of fibre and aspen by 0.6-18 g and fruiting was delayed by 4 days. Addition of aspen to bark or fibre resulted in similar yields, however, a mixture of fibre and bark was 0.7-19 g more than a mixture of fibre and aspen. Bran supplemented bark alone was unsuccessful as a fruiting medium, although upon addition of aspen, fruiting was observed and the yield was nearly equal to aspen or a mixture of fibre and bark (Table 7).

Growth rate of isolates at different vegetative temperatures.

Radial extension of mycelia growing on 2% malt-agar in petri-dishes were measured. Mycelial growth was suppressed at 15^{o} C - the reported optimal temperature for fruit-body induction - and at 35^{o} C. Optimum temperature for all isolates was within the range of 20-25^{o}C (Table 8). Therefore, in the next experiment, these two temperatures were compared using waste substrates.

Effect of temperature during vegetative run on subsequent yield.

Two chambers were set at 20^{o} C and 25^{o} C for the vegetative run followed by 15^{o} C fruiting temperature. In the three media, yields were similar at 25^{o} C and 20^{o} C (Table 9). Time taken for the first crop to mature was also similar, fruit-bodies were harvested after 34-37 days of inoculation.

Table 7. Mushroom yield on bark and fibre wastes with 20% (w/w) wheat bran supplementation.

Medium	Replicate (No.)	Time (range) to crop (Days)	Average yield (g)±S.D. (Total of both flushes)	B.E.(%)
As	5	36-38	21.0±3.3	80
AS, BARK (1:1)	5	46-48	18.4±7.6	70
AS, FIBRE (1:1)	6	45-47	9.0±7.7	30
			4<u(AS)-u(AS, FIBRE)<20*	
FIBRE	5	47-49	10.3±4.3	40
			5<u(AS)-u(FIBRE)<16	
			-1<u(FIBRE)-u(FIBRE,BARK)<-15	
FIBRE, BARK (1:1)	5	48-49	19.0 ±5.5	70
			-0.7<u(AS, FIBRE)-u(FIBRE,BARK)<-19	

* Confidence intervals (95%) comparing aspen with a fibre and mixture of aspen and fibre, other comparisions were insignificant. Experiment was repeated twice and the average yield of the two were tabulated.

Table 8. Average radial mycelial growth of isolates on malt agar at different temperatures.

Isolate	Temperature (^{o}C)			
	15	20	25	35
		(mm/day)		
DAOM-188690	2.6	5.0	6.8*	
DAOM-129030	5.6	7.8	8.2	
Malloch 8.12.73/8	5.2	7.6	7.1	
ECS-1669	5.5	7.7	8.4	
ECS-1673	-	4.2	6.3	-

*Underscored values indicate optimum temperatures. - signifies lack of mycelial growth.

Table 9. Mushroom yield at different temperatures during vegetative run.

Medium (with 20% [w/w] wheat bran supplementation)	Replicate (No.)	*Temperature(^{0}C) 20	25
		Average yield ± S.D.	
AS	6	16.2 ± 6.9	24.2 ± 5.4
SP	6	8.2 ± 6.2	10±3.8
PW	6	20.2 ±1.7	21.0 ± 2.4

*No significant difference between a medium at the tested temperatures (95% confidence interval). Experiment was repeated twice and the average yield of the two were tabulated.

Preliminary elemental analyses of pulp waste and mushrooms

Heavy metals in the pulp waste were present in very small amounts [Table 10]. Traces of cadmium (0.04 ug/g on dry weight basis) and relatively high amounts of macronutrients and Na were detected. Moisture content of the air-dry waste samples was approximately five percent.

Cadmium was undetected in two mushrooms and in three other samples low amounts (0.53 ug/g on dry weight basis) were found (Table 11). Considerable amounts of K, S, Na, Fe, Ca, Mg, Si and P were present in the mushroom samples. In comparision to pulp waste, mushrooms were higher in P and K. The N content of fresh pulp waste sample was 1100ppm and was 26740ppm in fresh mushrooms. The moisture content of mushroom samples was approximately 90 percent.

Table 10. Elemental composition of pulp waste.

Element	*Range (ug/g) (in oven-dry sample)	*Average (ug/g) (in oven-dry sample)	Estimated average (ug/g) (in air-dry sample with 5% moisture content)
**N	** 900-1300		1100
Al	124-135	130	26
As	1.5-1.6	1.55	0.31
Cr	1.60-2.00	2.00	0.4
Ca	135-142	139	28
Cd	0.033-0.047	0.040	0.0080
Fe	80-93	87	17
Hg	0.22-0.53	0.37	0.075
K	26-28	27	5.4
S	13-22	18	4
Mg	61-82	72	14
Mn	4.2-4.4	4.3	0.86
Na	46-47	46.5	9
Nb	45-47	46	9
P	9-9.2	9.1	2
Pb	0.4-0.6	0.5	0.1
Se	0.55-0.6	0.57	0.11
Sr	0.7-0.8	0.75	0.15
Th	0.5-0.7	0.6	0.12
U	1.1-1.3	1.2	0.24

Table 10 (Continued). Elemental composition of pulp waste.

Element	*Range (ug/g) (in oven-dry sample)	*Average (ug/g) (in oven-dry sample)	Estimated average (ug/g) (in air-dry sample with 5% moisture content)
Y	0.023-0.023	0.023	0.0046
Zn	1.0-1.2	1.1	0.22
Zr	0.14-0.26	0.4	0.08
Co	0.06-0.07	0.065	0.013
V	0.15-0.20	0.17	0.035
Ni	0.85-0.96	0.9	0.18
Mo	0.13-0.14	0.135	0.027
Ba	2.00-2.10	2.05	0.41

*ug/g in two oven-dry samples.
**ppm in two fresh samples.

Table 11. Elemental composition of *F. velutipes* mushroom.

Element	Average (ug/g) composition of oven-dry mushroom						Estimated average (ug/g in fresh samples with 90% moisture content)
	*A	"B"	"C"	"D"	"E"	Average	
**N	24500	28800	26800	29100	24500		26740
Al	0.32	0.50	0.42	0.24	1.75	0.65	0.0072
As			0.22	0.08	0.10	0.08	0.00088
Cr	0.008	0.54			0.51	0.21	0.0023
Ca	3.26	4.05	3.05	4.44	4.80	3.92	0.043
Cd		2.50	0.14	0.013		0.53	0.0058
Cu	0.22	0.18	0.15	0.44	0.30	0.25	0.0028
Fe	1.15	1.77	1.08	1.92	2.37	1.73	0.019
Hg		0.006	0.043	0.086	0.06	0.04	0.00043
K	199	378	69	413	322	276	3.06
S	1.48	1.75	1.32	4.74	9.7	4.00	0.044
Mg	10	21	3.00	18	24	15	0.16
Mn	0.040	0.076	0.021	0.067	0.076	0.056	0.0006
Na	8.40	10	1.45	43	17	16	0.18
P	52	80	17	111	15	52	0.60
Pb				0.01	1.1	0.22	0.002
Si	2.00	0.45	0.045	2.00	1.2	1.13	0.012
Se			0.020		0.36	0.076	0.0008
Sr	0.004	0.008	0.012	0.016	0.02	0.012	0.0001

Table 11 (Continued). Elemental composition of *F. velutipes* mushroom.

Element	Average (ug/g) composition of oven-dry mushroom						Estimated average (ug/g in fresh samples with 90% moisture content)
	*A	"B"	"C"	"D"	"E"	Average	
Th		0.10		0.007	1.18	0.25	0.0028
U					1.94	0.38	0.0043
Zn	0.30	0.50	4.18	1.84	0.45	1.45	0.016
Zr	0.009		0.033		0.09	0.026	0.0003
Co		0.004	0.035		0.14	0.035	0.0004
V					0.15	0.03	0.0003
Ni		0.11		0.13	0.72	0.20	0.0022
Mo	0.001		0.008	0.017	0.11	0.027	0.0003
Ba	0.074	0.057	0.026	0.015		0.034	0.0004

*Five replicates each of mushrooms "A" and "B", three of "C" and "D" and one of mushroom "E" were analyzed.

**ppm in fresh mushroom.

DISCUSSION

In all experiments, the major portion of the yield was obtained in the first flush. There was a decline in quantity and quality with time, possibly due to depletion of substrate nutrients and mycelial reserve materials. Commercial Japanese operations report a similar decline in yield with time (Tonomura, 1978). Yields with bran supplemented waste substrates and their mixtures, with the exception of spruce, were comparable to commercial operations in Japan where B.E. of approximately 47-64% were reported (Tonomura, 1978). From New Zealand, highest B.E. of about 22% with a New Zealand strain and 38% with a Japanese strain were reported (Chu-chou, 1983). With pulp waste, the highest B.E. reported for *P. ostreatus* mushrooms were approximately 79% (Muller and Gawley ,1983)). In the present study *F. velutipes* yield with pulp waste was higher (84% B.E.). Highest B.E. for the species is 100% as reported by Shiio *et al.* (1974). They described a vat cultivation method involving inoculation of surface with pieces of fruit-bodies in addtion to mixing the seeds (inoculum) with the substrate. However, attempts to induce fruiting on bran supplemented sawdust utilizing vats in this study were unsuccessful. Although mycelial growth was good, experiments were stopped a week after inoculation due to contamination. With the jars, B.E. close to that reported by Shiio *et al.* (1974) [96%] was obtained in the present work.

In the present work, all three substrates (i.e. aspen, spruce sawdust, and pulp waste) supplemented with bran were successfully utilized in the production of *F. velutipes* mushrooms. Mixtures of these materials also gave good results. Low yields with spruce were perhaps a result of higher lignin content in comparison with aspen (Browning, 1963). Aspen has 16% lignin as compared to 28% for spruce (Browning, 1963) and therefore, aspen is degraded faster than a softwood like spruce (Kirk,

1973). Also, water is better able to penetrate hardwoods than softwoods (Ander *et al.*, 1975), and this may result in a more favourable environment for fungal growth. Enhanced mushroom yields with pulp waste as compared with spruce may have been because the former medium is less compact, thereby resulting in increased aeration and porosity and a better environment for fungal growth. Mushroom yield with bran supplemented pulp waste equaled the best yields obtained with bran supplemented aspen and the effects were beneficial rather than negative.

It was found that additional nutrients enhanced mushroom yields on all substrates tested. This perhaps is evidence that naturally occurring nitrogen in the wood is low. Woody tissues have only 0.03-0.10 % nitrogen (Cowling and Merril, 1966), but there is a large mass of substrate material available in the tree, that exceeds the substrate available in the laboratory. Better yields on aspen (0.10 % nitrogen) than spruce (0.05% nitrogen) may also be due to higher nitrogen content of the former.

Rice bran is the nutrient supplement currently being used in commercial cultivation of *F. velutipes* in Japan (Tonomura, 1978). Wheat bran was chosen because it is readily available and cheap in Canada. Also it has a 14.8% protein content as compared to 13.5% for rice bran (Pearson, 1970). Han *et al.* (1981) reported better yields of shiitake mushrooms with wheat bran as the nutrient supplement in comparision to rice bran. Increase in yield (0.8-12 g) with the increase in percent bran of the media mixture from 20% to 50% was evident and was clearly due to enrichment and an increase in nitrogen content of the substrate (Table 4). Similar results were reported by Royse and Bahler (1986) for shiitake mushroom. Barley grain and coomings were other nutrient supplement tested that were readily available in Thunder Bay. Jars supplemented with barley had very dense mycelial growth and like pure bran failed to produce fruit-bodies. During the course of these experiments, it was observed that jars with dense mycelial growth failed to fruit. This may indicate that nutrient stress might play an important role in initiating fruiting (pH changes, toxic staling products accumulate [Hawker, 1957]). Both coomings and bran gave

similar yields, although fruiting on the former was delayed by 5 days. The time period to crop is important factor in successful mushroom cultivation (Tonomura, 1978).

The optimum temperature for mycelial growth differs with the strain (Aschan, 1958, 1960; Croft and Simchen, 1965). Optimum temperature for four of the isolates was close to 25° C. This is in agreement with optimum temperature of 24° C reported by Aschan (1958). Isolate Malloch however exhibited optimal growth at 20° celsius. The upper limit of growth was $\leq$ 35° C and at 15° C the mycelial growth was suppressed. Temperatures favourable for fruit-body production were unfavourable for mycelial growth (Kinugawa and Furukawa, 1965). Mushroom yields were similar at both 20° C and 25° C and significant differences between the three substrates at these temperatures were undetectable, because of wide variations in yield within replicates. Statistically significant differences between various moisture levels were also undetected. However, need for proper moisture levels was clearly evident as moisture levels at or below 60% during preliminary experiments resulted in reduced yields. Considering the time period to crop (first harvestable flush) as an important factor in mushroom cultivation, fruiting at 25° C and on substrates having 150% moisture content would result in a shorter cultivation cycle, Wastes such as bark and fibre were utilized in the production of *F. velutipes* mushrooms, however, the cropping time was 48-50 days as compared to 35-36 days with aspen, spruce or pulp waste as substrates.

Isolates 188690 and ECS-1673 fruited whereas Malloch 8. 12. 73/8, 129030 and ECS-1669 failed to produce fruit-bodies. The fruit-bodies of ECS-1673 were very viscid. Cytological examination of the two fruiting isolates revealed that 188690 possessed true clamps while ECS-1673 was simple septate and homokaryotic. At present no explanation can be offered as to why the three isolates failed to fruit.

Although external factors affecting fruiting have been investigated in *F. velutipes* (Plunkett, 1956; Aschan, 1958, 1960b), in *A. brunnescens* (Ingratta, 1984) and in *L. edodes* (Royse and Bahler,

1986), internal factors regulating fruiting remain largely unknown. Raper (1966) stated that fruiting competence is heritable. A dikaryon fruiting well may be composed of two "good" fruiting strains, or "one good and one intermediate" strain, or "one good and one poor" strain. A dikaryon carrying two "poor fruiting strains" may fruit sparsely or not at all. This may perhaps explain the performance of isolates in the present work.

Variation in yields from one experiment to another were frequently observed. Review of past literature revealed that this variation is quite common. Raper and Krongleb (1958) reported tremendous variability in all aspects of the fruiting process in normal strains of *Shizophyllum commune* . Royse *et al.* (1985) have reported variation within the replications and among experiments for *Lentinus edodes*. Often workers overlook this kind of variation in their data (Raper, 1966). Aschan (1958) commented that quantitative comparision of fruit-body production under different conditions in *F. velutipes* presented great difficulties. Probable causes of variation in the present study may have been lack of humidity control in the chambers, power failures, internal (viruses, mycoplasmas) and/or external (weed fungi, mites) contamination of the mycelium.

Elemental analysis of pulp waste and mushrooms provided an indication of the effect of this substrate on redistribution of elements in the fruiting-bodies. Heavy metals were present in very low concentrations in pulp waste and with the exception of Hg, the same was true for the mushroom samples (Table 10 and 11). In pulp waste, the amount of heavy metals were within the limits (for application of wastes for agricultural purposes) proposed by Chaney (1973). There have been few investigations of Cd and Pb content in substrates and plants, especially mushrooms. However, variations in heavy metal content of pulp wastes, climatic conditions, soil properties, and varietal differences in crop metal absorption make coordination of results difficult. Although there is no legislation controlling the amount of Cd present in foods (Thomas *et al.*, 1972), Brockway reported levels $\leq$1ppm in plants as non-hazardous.

Cadmium level in *F. velutipes* mushrooms was 0.53ppm, on a dry weight basis and 0.0058ppm on fresh weight basis (Table 11). The lead in Food regulations set a limit of 2ppm for vegetables; statutory limit for Pb is 7 ppm in Canada (Thomas *et al.*, 1972). In *F. velutipes*, Pb was within these limits (0.22ppm) [Table 11]. Average level of Hg was close (0.04ppm) to the permissible levels (0.05ppm) for Hg in foods set by WHO (National Research Council Canada, 1979) and was $\geq$ 0.05ppm in two mushroom samples. Local studies on this subject are rare, but one investigation reports heavy metal content (on dry weight basis) of feather moss(*Pleurozium schreberii*) growing under natural conditions, 50-200 km from Thunder Bay (Rinne, 1977). In comparison to this study, on a dry weight basis, Zn, Pb, Ni, Hg, Fe and Cu levels were lower in mushrooms and Cd levels (0.53ppm) were comparable to moss (0.5-0.6ppm).

The main chemical processes presently used in pulp production at Great Lakes Forest Products (Thunder Bay) are sulphite and kraft [Donavin (pers. comm., 17 August 1987)]. Commercial pyrites may contain metal sulphides of Cu, Zn, Pb, etc. which can contribute to the metal composition of the pulp waste. However, present solid waste treatments lower their amount substantially (Bruely, 1974). White rot fungi are known to oxidize persistent environmental pollutants to CO_2 and extracellular enzymes degrading lignin or a similar enzyme system has been suggested in the biodegradation of organic chemicals (Bumpus *et al.*, 1985). The decomposition of byproducts of pulping process by white rot fungi such as *F. velutipes* would therefore cause a degradation of organics present in the byproducts, rendering them more suitable for mushroom production and also providing a means for their safe disposal.

Although this study involved small-scale experiments, the possibility of utilizing wastes like sawdust, pulp wastes, bark, and fibres in the production of *F. velutipes* mushrooms was demonstrated. Further research is needed to select and breed the best strains for commercial operation and to insure that ligninocellulosic wastes are safe for use in this manner.

CONCLUSIONS

Local forestry residue such as sawdust of aspen, black spruce, pulp waste, bark and fibre effectively served as substrates in the production of *F. velutipes* mushrooms. With isolate 188690, a B.E. close to 100% was obtained under the following regime: 1) incubation temperature of 25^{o} C during the vegetative run for 2-3 weeks, 2) substrate moisture contents ranging from 125- 150% based on oven-dry weights, 3) wheat bran supplements to the wood, and 4) a cropping time of 36-49 days at 15^{o} C as the fruiting temperature. There was significant increase in B.E. when the amount of wheat bran in the substrate was doubled.

Success of these small-scale experiments supports the conclusion that wood wastes in Canada might be suitable substrates for growing these mushrooms provided there are no hazardous chemicals present in the final product. Low amounts of heavy metals in both pulp waste and mushrooms (with the exception of Hg in mushrooms) growing on the wastes support the view that these wastes may be utilized in mushroom production although careful monitoring would be necessary. Various organic chemicals may present a potential health problem although the white rot fungi are known to decompose organic pesticides. Further studies are needed to study this aspect and commercial feasibility needs to be evaluated on a large scale.

CYTOLOGY OF *FLAMMULINA VELUTIPES*

CYTOLOGY

INTRODUCTION

Hymenomycetes exhibit considerable variation in nuclear behavior. This diversity of nuclear behavior has been examined in many species, especially in relation to the basidiocarps. These include *Coprinus ephemerus* Fr. (Sass, 1929), *Schizophyllum commune* Fr. (Ehrlich and McDonough, 1949; Bakerspiegel, 1959), *Fomes annosus* (Fr.)Karst. (Wilson, Miller, and Griffin, 1967), *L. edodes* (Nakai and Ushiyama, 1977), *Collybia maculata var. scorzonerea* (Fr.)Gillet (Huffman, 1968), and a number of other species in many earlier papers reviewed by Olive (1953).

There have been no recent cytological studies dealing with nuclear division in the basidium nor vegetative mycelium of *F. velutipes*. The objectives of this study were to re-examine the nuclear life history of *F. velutipes* with traditional and more modern staining procedures and to verify the tetrasporic nature of the basidia in some of the isolates used in the mushroom culture study. Also, the mushroom culture study of this species has revealed tremendous variability among different isolates and this prompted a cytological investigation of isolates and their nuclear behaviour as the probable cause of this variation.

LITERATURE REVIEW

The nuclear cycle in most hymenomycetes follows a similar pattern. In heterothallic species such as *F. velutipes*, two compatible primary mycelia combine to form a secondary or dikaryotic mycelium. This secondary mycelium later develops into a basidiocarp and a hymenial layer with binucleate basidia. The two nuclei in the basidium fuse and the fusion nucleus undergoes meiosis. This results in

four presumably haploid nuclei which migrate into basidiospores. Variations on this pattern of nuclear behavior exist among species.

Nuclei during the life cycle of a fungus often differ in appearance. The nuclei may be oval, spherical, or elliptical in shape, may be located centrally in a cell or may occur close to a cell wall, may be "expanded" or "constricted", or they may differ in diameter (Chang and Hayes, 1978). Among species, meiotic-divison in the hymenial layer may be synchronous or asynchronous; it may occur within an intact nuclear envelope or the envelope may disintegrate; a postmeiotic mitotic division may occur in the basidium, sterigmata, or in the basidiospore and the basidiospore may be uni- or binucleate depending on the species (Sass,1929; Duncan and Macdonald, 1970; Wilson, Miller, and Griffin; 1967; Huffman, 1968; Setliff *et al.*, 1974; Nakai and Ushiyama,1977). The number of basidiospores per basidium may vary in a species, and in a number of *Agaricus* species, variation in spore number has been observed on basidia from the same basidiocarp (Kerrigan and Ross, 1987). Similar observations have also been reported for *Coprinus lagopus* (Mclaughlin, 1982). These species were previously described as being tetrasporic as has *F. velutipes* (Aschan, 1954; Buller, 1958; Takemaru, 1961).

While there has been considerable work relating to the basidial cytology, divisions in the vegetative hyphae have not received similar attention. This may be because somatic divisions would occur more rapidly (Chang and Hayes, 1978), and last only for a few minutes (Thielke, 1972) whereas a 16 hr time span of meiosis was reported for *Coprinus* (Raju and Lu, 1970).

The somatic divisions were a subject of dispute in the past. Some workers considered these divisions to be amitotic (Bakerspigel, 1959; Saksena, 1961). These reports described nuclear division to be a simple constriction in the middle of the nucleus without the presence of spindle, centrioles, or metaphase plate. Other workers reported this division to be typically mitotic on the basis of ultrastructural

studies that showed the presence of spindle, centrioles, and individual chromosomes (Lu, 1964; Girbardt, 1968). Olive's (1953) comment that " Mitotic divisions occurring in the vegetative phase of the fungi are essentially the same as in higher organisms, although the details are frequently obscured by the small size of the nuclei " has been supported by some work in this area (Aist and Wilson, 1968; Brushaber and Jenkins, 1971). In order to overcome the problem of small nuclei and small chromosomal size in fungi, a number of studies have focused on measuring nuclear DNA utilizing techniques such as absorption spectrophotometry and fluorescence photometry (Peabody *et al.*, 1978; Peabody and Peabody, 1983). The latter technique has been suggested as being superior as it detects substances present in very low concentrations and is ideal for observing and measuring small fungal nuclei in the mycelium (Peabody and Peabody, 1983). DNA in spores and mycelia of fungi (Williamson and Fennell, 1975), viruses in HeLa cells (Russell *et al.*, 1975) have been stained with 4', 6 diamidino'-2- -phenylindole (DAPI), a stain which forms a fluorescent complex with DNA. In the present study, mycelium of *F. velutipes* isolates was examined using DAPI as a fluorescent probe for DNA at the cellular level.

Few studies have focused on the cytological aspect of *F. velutipes*. Oidia or the asexual spores of the species were examined by Brodie (1936). He reported that uninucleate oidia were produced on both mono- and dikaryotic mycelium. In dikaryotic mycelium they were borne on haploid branches and these haploid branches were presumably produced by separation of nuclei in the dikaryon. So, half of the oidia were of the same sex compatibility group as one of the parent mycelium and the other half were the same as the other parent mycelium constituting the dikaryon. Later Aschan (1952) observed that oidia were produced in chains mainly on the aerial mycelium and oidia of a single chain had the same genotype. Kemp (1980) using morphological markers established that oidia were formed in chains on clumps of aerial hyphae behind a clamp connection. He further noted that some dikaryons formed oidia of one genotype only. He observed a homing reaction of the hyphal tips towards an oidium when it was placed in contact

or up to 30um distance from a dikaryotic or monokaryotic hypha. He suggested that formation of a haploid branch rather than a dikaryotic branch on a dikaryon depended on the length of time a nucleus remains trapped in a clamp connection and on the prevention of its migration into a side branch. In a primordium, the stipe and pileus cells were binucleate, and were found to be multinucleate in mature fruit-bodies, although the lamellar trama cells remained binucleate (Wong and Gruen, 1977). The nuclear condition of basidiospores has been been reported as uninucleate by some (Raper, 1978) and binucleate by others (Duncan and Galbraith,1972). This characteristic is important to both taxonomists (Singer, 1967) and systematists (Kuhner, 1977), cytological examination of spores was important. The basidiocarps were examined with the scanning electron microscope to observe any deviations in spore number from the described tetrasporic condition. A clear cytological perspective of an organism provides the basis of understanding other aspects of its biology - particularly genetics and pathology.

MATERIALS AND METHODS

Basidiocarps obtained from the mushroom culture experiments were used in the cytological work. Nuclear behavior in all life-cycle stages was followed by light microscopy. The number of sterigmata and basidiospores on basidia were examined with a scanning electron microscope (S-570, Hitachi, Japan) operating at 12 KV to note any anomaly in the life-cycle.

Light microscopy

Oidial suspensions were obtained by flooding a week-old plate of actively growing mycelium on 2% malt agar with sterile 1% glucose solution. The asexual spores or oidia were loosened with a sterile L-shaped glass rod and the suspension was sucked through a folded pad of sterile cheese cloth. A drop of suspension was placed on a cover glass and air dried. The oidia were fixed in Carnoy's fixative (three parts ethyl alcohol to one part glacial acetic acid) for 10 min, rinsed in distilled water for 2-3 min, hydrolyzed in 5-6N HCl for 10 min, stained with giemsa stain for 30 min, rinsed in 0.15 M phosphate buffer (pH 7) for 5 min and mounted in phosphate buffer. Germinating oidia, obtained by placing air dried oidial suspension drops on a cover glass and incubating in a moist petri dish at 25^{o} C for 12-16 hr, were stained by a similar method and observed with a Zeiss microscope (Carl Zeiss, Ultraphot II, West Germany) for nuclear content. Fresh basidiospores were collected on a glass slide. They were either stained immediately by the HCl-giemsa technique or were incubated in a moist chamber for germination. Best results were obtained by staining the ungerminated and germinated spores overnight and destaining the slides in phosphate buffer for 30 min. Vegetative mycelia of all five isolates were grown on a sterile

No. 1 cover glasses or cellophane pieces (22 x 22 mm) placed on 2% malt agar plate and inoculated with an agar plug in the center of a cover glass or cellophane pieces. The cultures were incubated at 25^{o} C and after 7-10 days, when sufficient mycelial growth had occurred, the cover glass was removed from the plate and air dried. Observations were also made with the light microscope bright field and phase contrast optics.

Mycelia were also stained with DAPI (4', 6 diamidino'-2-phenylindole) stain. The stain was prepared by adding 5 ul DAPI stock solution in 20 ml buffer. Buffer (pH 4.4) was prepared by adding 11.25 ml citric-acid solution (prepared by diluting 1.05 g citric-acid to 50 ml distilled water) to 8.75 ml (0.2 M) $Na_2 HPO_4$ and diluted to 500 ml solution. Mycelial sections were fixed in 70% ethyl alcohol for 30 minutes. They were stained for 90 minutes. Next they were rinsed quickly in buffer and mounted in buffer (Williamson and Fennell, 1975). Similar procedure was followed for DAPI controls with the omission of DAPI staining. Photomicrographs were taken on Plus-X film (Kodak, Toronto, Canada) with a Zeiss microscope equipped with a camera. The film was developed in Kodak D-76 developer.

Plastic embedding techniques were used to study basidiocarp tissue. Sections of the basidiocarp tissue (stipe and gills) were dehydrated and embedded in glycol methacrylate (Feder and O'Brien, 1968). Stipe tissue and longitudinal and transverse sections of the basidia (2-4 um thick) were cut with glass knives mounted on a JB-4 microtome (Sorvall, USA). Sections were lifted with the forceps and placed in a drop of water on a glass microscope slide. After drying, the sections were stained with iron-haematoxylin for 4-10 min.

Scanning electron microscopy

A technique involving OsO_4 vapor fixation and acetone vapor diffusion dehydration was employed for examining basidiocarp tissue with the SEM operating at 12 KV (King and Brown, 1983). After fixation, the samples were transferred to a critical point drying apparatus and dried after five exposures of 5 min duration each in the transitional fluid (CO_2). All dried samples were mounted on the SEM mounts and were sputter coated with gold before examination. Photomicrographs were taken on Ilford Pan F, 50 ASA film with an automatic camera (Mamiya, Japan). Samples were taken from two different basidiocarps and a total of 100 basidia were examined in each. In the basidia, only the top view of basidia bearing sterigmata or basidiospores was counted and ambiguous basidia were deleted from the count. Both centre and near margins of a sample were examined as basidia at the margins were collapsed making observation difficult. About 100 basidia in each of two basidiocarps of isolate 188690 were examined. In each basidiocarp section, 10 basidia were scored from 10 random fields of view. Areas near the margins, at the margins and at the centre of the sample were scanned.

RESULTS

RESULTS OF LIGHT MICROSCOPY

Nuclear behaviour in the basidiocarp

In the lamellae, tramal cells were binucleate and the nucleolus was clearly seen (Fig.1) . Central cells were broad with large vacuoles and usually had a dense cytoplasmic band with two nuclei either in the middle of the cell or on one side. Subhymenial cells on margins were comparatively thinner in diameter and had densely staining granules. All the hyphal cells were clamped. In the hymenial layer, conical cystidia were interspersed with the basidia. These had a large central vacuole and were 40 x 10 um in size (Fig. 2). Young basidia were initially binucleate and the nuclei occupied the midregion of the basidium (Fig. 3). After karyogamy, the fusion nucleus migrated to the upper end of the basidium (Fig. 4). The spherical fusion nucleus appeared about twice the size of nuclei in young basidia.

The five classical stages of prophase I were difficult to identify. At pachytene (Fig. 5) the chromosomes were elongated. Although the chromosomes achieved maximum elongation at this stage, it was difficult to count them. Later the chromosomes seemed to shorten and thicken at diplotene (Fig. 6). Progressive stages of metaphase and anaphase I were observed. At an early stage of metaphase 1, two spindle pole bodies (SPBs) were seen as densely staining dots on opposite sides with the chromatic mass distributed between them (Fig. 7). Later disjunction of chromatids between the two spindle pole bodies

Nuclear characteristics and meiosis of *F.velutipes* isolate 188690. Magnification of Fig. 1-14: X 2 000.

Figure 1. Binucleate (arrow a) tramal cells. Nucleolus (arrow b) is visible.

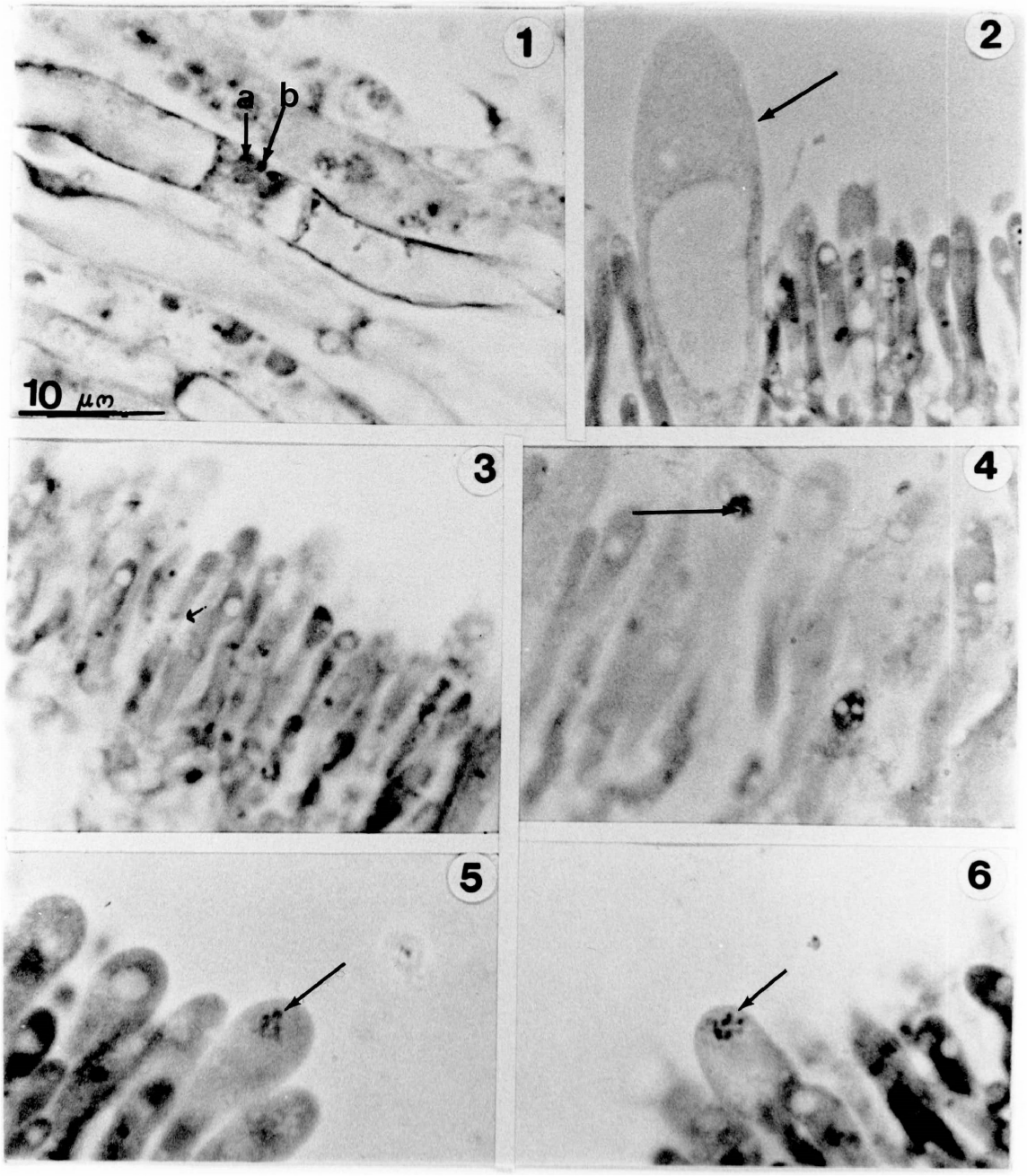
Figure 2. Cystidium (arrow) in the hymenium.

Figure 3. Young basidium with nuclei (arrow) in interphase.

Figure 4. Single diploid nucleus (arrow) in an basidium.

Figure 5. Pachytene, prophase I. Chromosomes (arrow) appear elongated.

Figure 6. Diplotene, prophase I. Chromosomes (arrow) appear condensed.


1
a
b
10 µm
2
3
4
5
6

was clearly seen at anaphase 1 (Fig. 8). The disjunction occurred at a plane obliquely transverse to the longitudinal axis of the basidium (Fig. 8 and 9). In this plane, chromosomes were observed in "parallel rows" around a central clear zone (Fig. 9). At telophase I, the chromatin was frequently observed (Figs. 10, 11, and 12). Stages between telophase I and telophase II were not be seen. This perhaps was due to the fact that the second division was very short in time span as compared to the first division. The resulting four nuclei of the second division (Fig. 13) migrated to the middle part of the basidium (Fig. 14) and stayed there during sterigmata formation. The migrating nuclei were elongated and the nucleoli were seen with greater clarity as compared to the condensed state of the resting nuclei (Figs. 14 and 15). A large vacuole filled the basidium as basidiospores developed (Fig. 16). As the spores matured, the nuclei migrated synchronously through the sterigmata and were tear-drop shaped (Fig. 17). A third division (post meiotic mitosis) in the attached basidiospore resulted in binucleate basidiospores (Fig. 18).

The stipe tissue contained two types of cells. The outer cells were loosely interwoven with frequent clamp connections and yellowish cytoplasm (Fig. 19). Inner cells were more elongated, parallel in arrangement, more compact, and multinucleate (Fig. 20). From 3-12 nuclei were found per cell. Some of these hyphal cells had very dense, dark granules. Fusion between two cells was frequently seen. The majority of these inner cells were broad with thinner hyphae interspersed between them.

Nuclear content of mycelium and spores

Hyphae of isolate Malloch 8. 12. 73/8 were mostly dikaryotic and averaged 2.05 nuclei per cell (Table 12). True clamps were observed at each point of septation (Fig. 21). In some of the cells the two nuclei were laterally paired but in some others, two separate pairs of nuclei were observed arranged in

Nuclear characteristics and meiosis of *F.velutipes* isolate 188690:

Figure 7. Meta- anaphase I. SPBs (arrow) on opposite sides of chromatin mass.

Figure 8. Later stage of anaphase I (arrow).

Figure 9. Top view of anaphase I. "Parallel row" arrangement of chromatin (arrow a) around cylindrical clear space (arrow b).

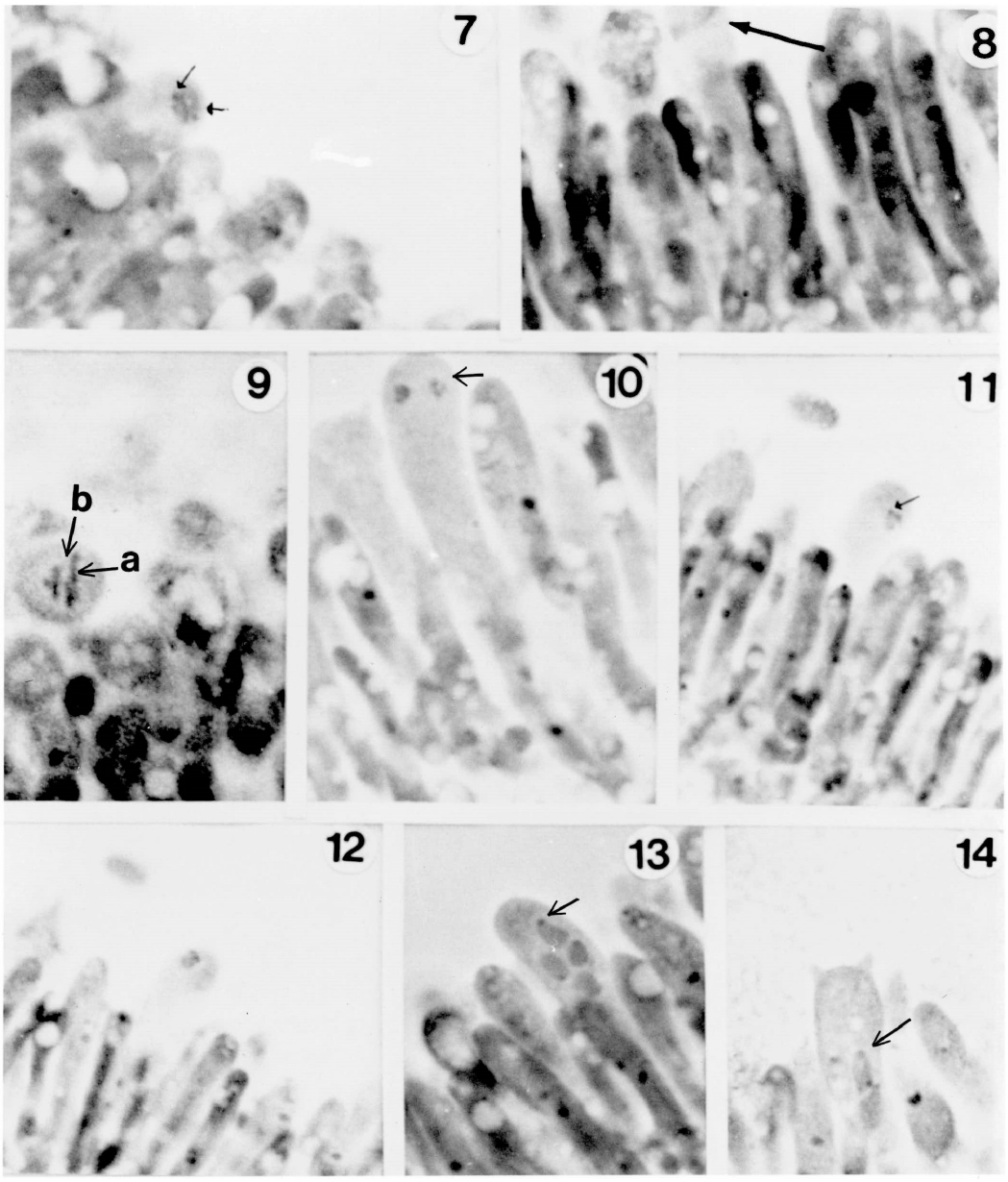
Figure 10. Telophase I (arrow).

Figure 11. One lunate nucleus of telophase I stage visible around electron opaque area (SPB). Nucleolus is visible (arrow).

Figure 12. Same basidium with the other nucleus in focus.

Figure 13. Four nuclei resulting from second division. Nucleolus at the tip of beak-like projection of downward migrating nuclei (arrow).

Figure 14. Nuclei at the base of the basidium (arrow) as sterigmata are initiated.


7
8
9
b
a
10
11
12
13
14

Nuclear characteristics and meiosis in *F.velutipes* isolate 188690. Magnification of Fig. 15-18: X 3 800, Fig. 19-28: X 550.

Figure 15. Migration of nuclei towards the apical end of the basidium.

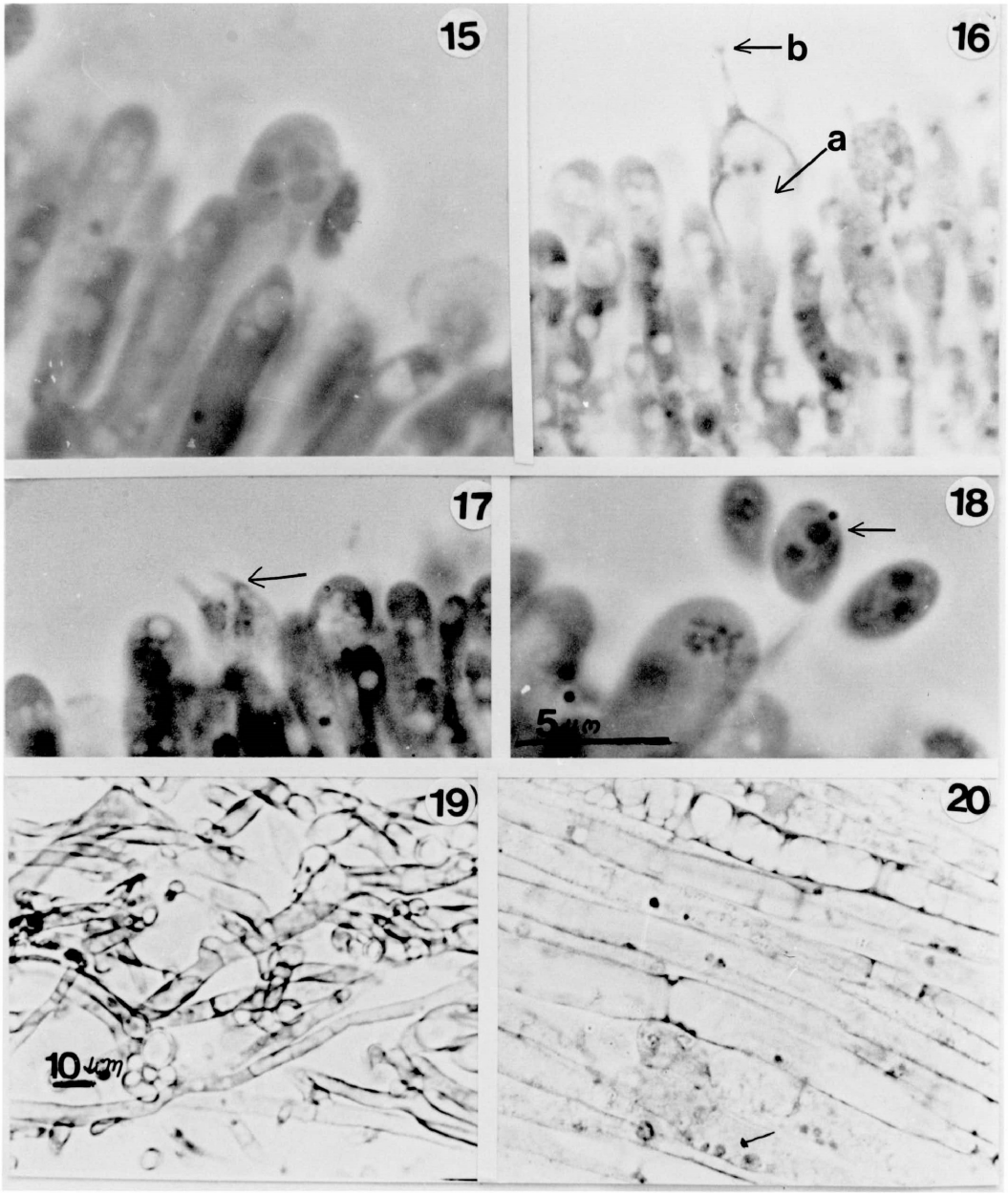
Figure 16. Vacuolation (arrow a) in the basidium and development of basidiospores (arrow b)

Figure 17. Migration of nuclei through sterigmata (arrow).

Figure 18. Binucleate (arrow) basidiospores.

Figure 19. Clamped outer, loose, yellow hyphae in the stipe.

Figure 20. Inner stipe cells in multinucleate (arrow) condition, isolate 188690.


15
16
b
a
17
18
5 μm
19
10 μm
20

two pairs. The apical end of some hyphal cells was swollen and had two to six nuclei in it (Fig. 22). Shiny oil-like globules were observed in all five isolates. Some of the cells had densely staining granules (Fig. 22 and 28). Many nuclei were elongated and with a beak like extension at one end; presumably these were migrating nuclei (Fig. 21, 35 and 38). The other nuclei were oval to spherical and condensed in appearance (Fig. 21).

Isolate 188690 was different from the other isolates in possessing both true and false clamps (Fig. 23). Some of the cells were dikaryotic but majority were multinucleate and averaged 2.2 nuclei per cell (Table.12). Often anucleate hyphal cells were observed close to the multinucleate ones.

Mycelium of isolate ECS-1673 was simple septate and multinucleate and averaged 2.45 nuclei per cell (Table 12). Densely stained granules were more abundant in the hyphal cells of this isolate (Fig. 28). Similarly hyphae of isolate 129030 were simple septate (Fig. 25), predominently multinucleate (Figs. 34, 35, and 39), with an average of 3.65 nuclei per cell (Table 12). The number of nuclei ranged from 1-9 and 25% of the cells were binucleate (Figs. 36 and 38) with laterally paired nuclei. Often migration of beaked nuclei into lateral branches was observed (Fig. 38).

Fragmentation of hyphal branches into oidia occurred mainly on the aerial hyphae. The oidia were predominently uninucleate, oidial chains often had bi- or trinucleate cells which were in the process of fragmenting (Fig. 25). Isolate ECS-1669 was similar to isolate 129030 and ECS-1673 (Fig. 26). The cells were simple septate, averaging 1.9 nuclei per cell. About 50% of the cells were uninucleate, 30% were binucleate and others had 3-6 nuclei per cell. Oidial formation and uninucleate oidia were observed in this isolate along with all others (Fig. 27). Bridges between the hyphae were clearly seen

Table 12. Nuclear and cultural characteristics of *F. velutipes* isolates.

Characteristic	188690	Malloch 8. 12. 73/8	129030	1669	1673
Number of nuclei/cell	2.2	2.05	3.75	1.9	2.45
Septum	clamped	clamped	Simple	Simple	Simple
Appearance	yellow less fluffy	yellow fluffy	white fluffy	white fluffy	yellow less-fluffy
Yellow particles	+[a]	+	+	_ [b]	+

[a] + present, [b] - absent.

Mycelial nuclear condition of *F.velutipes* isolates.

Figure 21. Dikaryotic cells of isolate Malloch 8.12.73/8. True clamp with a nucleus (arrow).

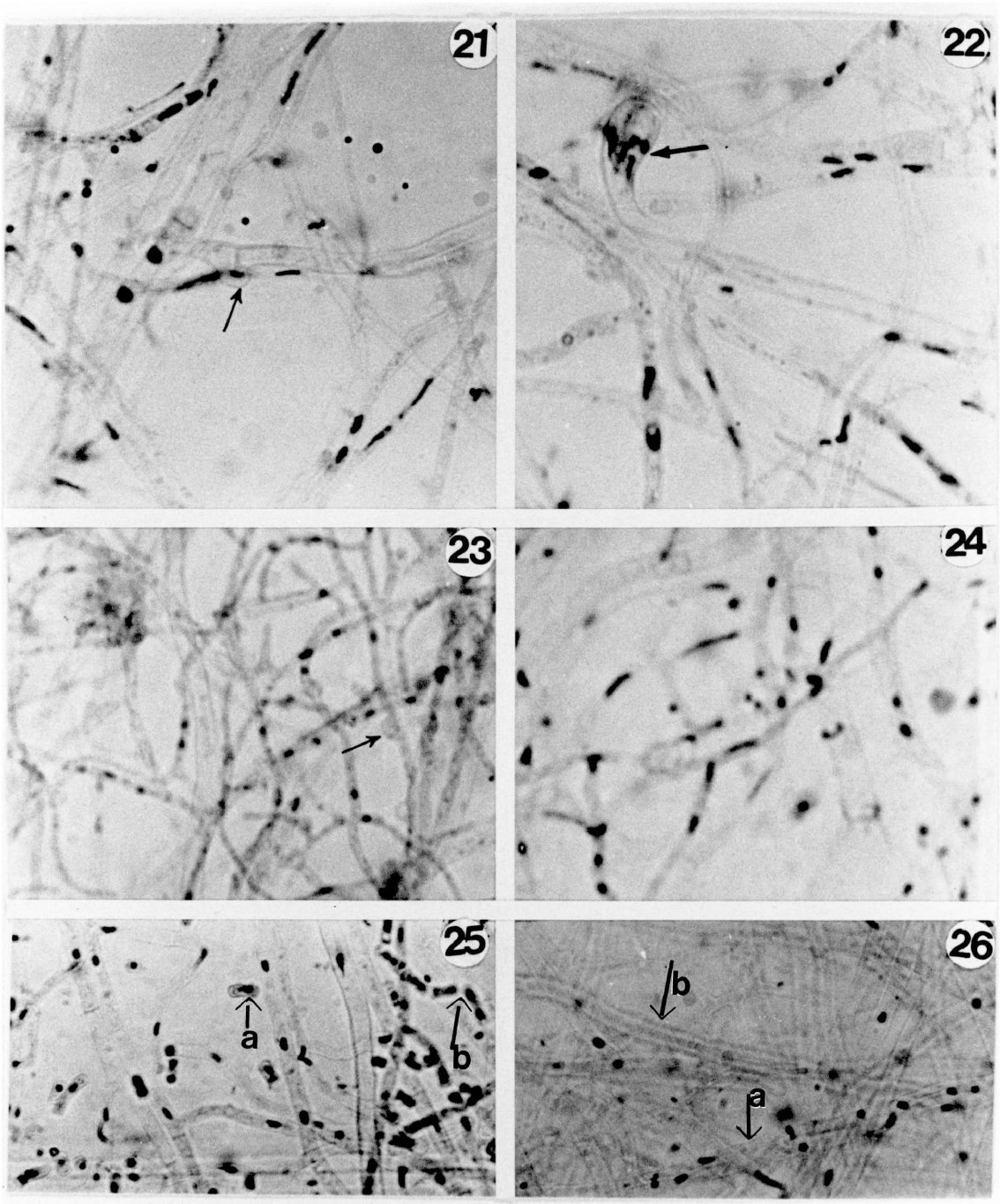
Figure 22. Club shaped, multinucleate apical end of isolate Malloch 8.12.73/8 (arrow) .

Figure 23. Dikaryotic and multinucleate cells of isolate 188690. True clamps (arrow).

Figure 24. Simple septate, multinucleate cells of isolate 129030.

Figure 25. Uninucleate oidia (arrow a) and oidial branches (arrow b) of isolate 129030.

Figure 26. Multinucleate (arrow a) and anucleate (arrow b) cells of isolate 1669.


21
22
23
24
25
a
b
26
b
a

in all of them, sometimes nuclei migrating through them were visible. The nuclei of hyphae stained with DAPI fluoresced pale blue in all isolates. In addition, a number of brightly fluorescing yellow particles were observed (Fig. 40). The frequency of these particles was highest (visual determination) in isolates Malloch 8. 12. 73/8, 129030 and 188690 (Table 12). In these isolates, the yellow particles were scattered in the cytoplasm and sometimes surrounded the nuclei. In anastamosing hyphae, they were present in the hyphal bridges and were observed in the clamps and walls of some empty cells fluoresced yellow. Some of the particles were larger than others and the particles were present in only some of the hyphal cells in a mycelium. Isolate ECS-1673 had very few particles and they were totally absent in the non-fruiting isolate ECS-1669. The controls failed to fluoresce.

Freshly shed basidiospores of isolate 188690 had shiny oily appearing globules. The spores were 3.8-6.8 um X 2-3 um in size. About 97% of the spores were binucleate, 2% were uninucleate and 1% were trinucleate (Table 13). The spores on germination seemed to swell before forming a germ-tube at one or both the ends (Fig. 29). The germ tube remained aseptate and multinucleate (2-6 nuclei per cell) by 100 um length.

Oidia were cylindrical and uninucleate on both binucleate and multinucleate isolates. They were 3.8-9.2 um X 1.5-4.8 um in size. The nucleus was usually central in position with two or more vacuoles in an oidium. On germination, thin germ-tubes were formed from one, two or three points (Fig. 30 and 33). In some of the oidia, after the germ tube had formed at one end, a thin-walled vesicle was formed (Fig. 32). The cytoplasm of the oidium moved into this vesicle (Fig. 33) leaving the oidia empty. One or more germ-tubes were now formed at the apical portion of this vesicle. Frequently, fusion between two adjacent oidia was observed (Fig. 31).

Table 13. Nuclear condition of basidiospores in isolate 188690.

Number of nuclei	1	2	3	Total
Number of spores	18	872	12	902
Frequency (%)	1.99	96.67	1.33	100

Nuclear condition of the spores and mycelia, and the germinating spores of *F.velutipes* isolates. Magnification of Fig. 29 : X 1 300, Fig. 30 : X1 500, Fig. 31-33 : X 800.

Figure 27. Uninucleate oidia and oidial fragments of isolate 1669.

Figure 28. Mycelium of isolate 1673 with dense granules (arrow).

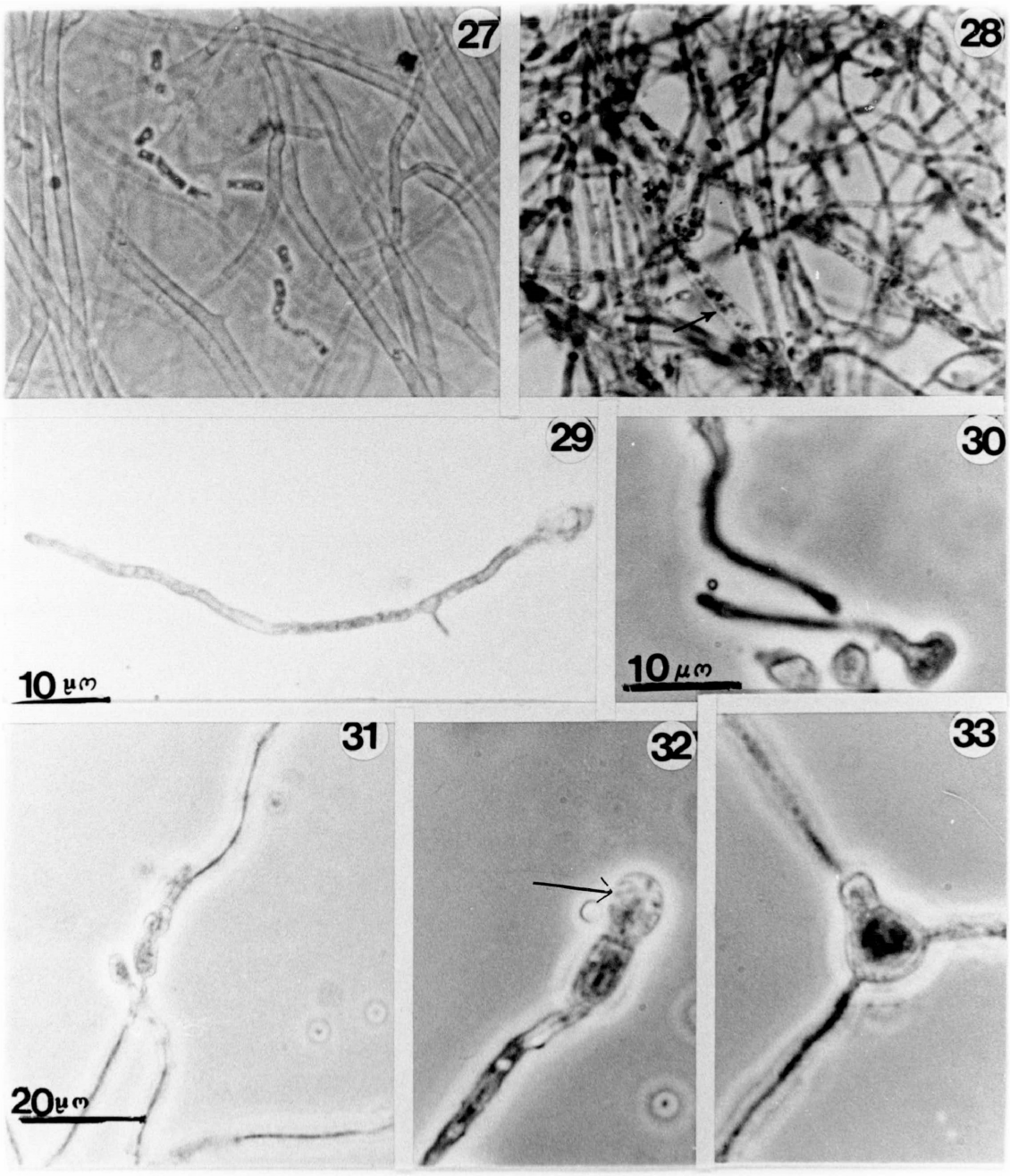
Figure 29. Germinating basidiospore, isolate 188690.

Figure 30. Germinating oidia, isolate 188690.

Figure 31. Fusion between two germinating oidia (arrow), isolate 188690.

Figure 32. Formation of vesicle (arrow) from opposite side of the germ tube in an oidium, isolate 188690.

Figure 33. Three point germination of an oidium, isolate 188690.


27
28
29
10 µm
30
10 µm
31
20µm
32
33

Nuclear characteristics of *F.velutipes* isolate 129030. Magnification of Fig. 34 - 39: X 1 900.

Figure 34. Uninucleate hyphal cell.

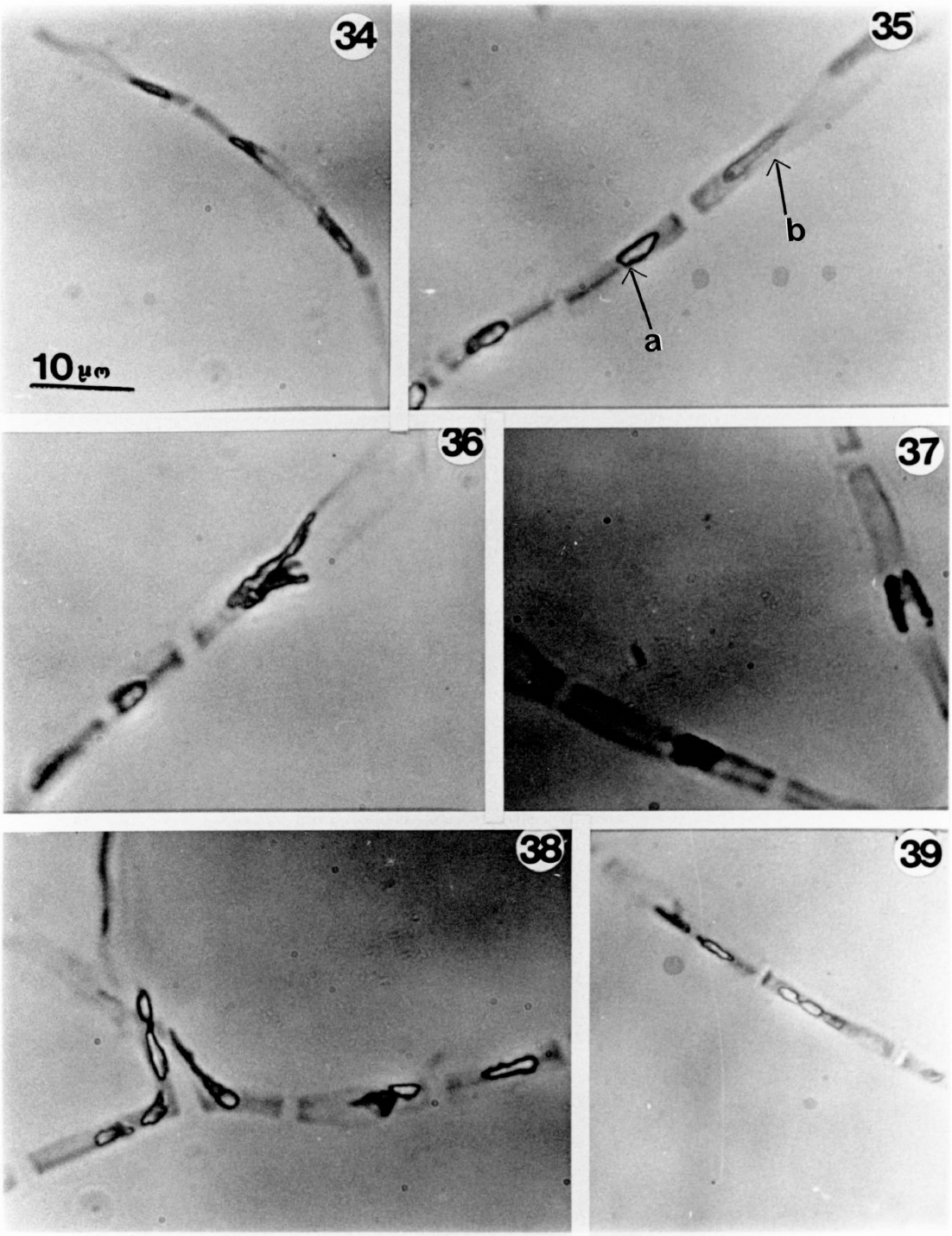
Figure 35. Uni- (arrow a) and binuclate (arrow b) cells.

Figure 36. Laterally paired migrating nuclei.

Figure 37. Laterally paired nuclei.

Figure 38. Multi- and binucleate condition and migration of nuclei to hyphal branches.

Figure 39. Multinucleate hyphal cells.


34
10 µm
35
a
b
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37
38
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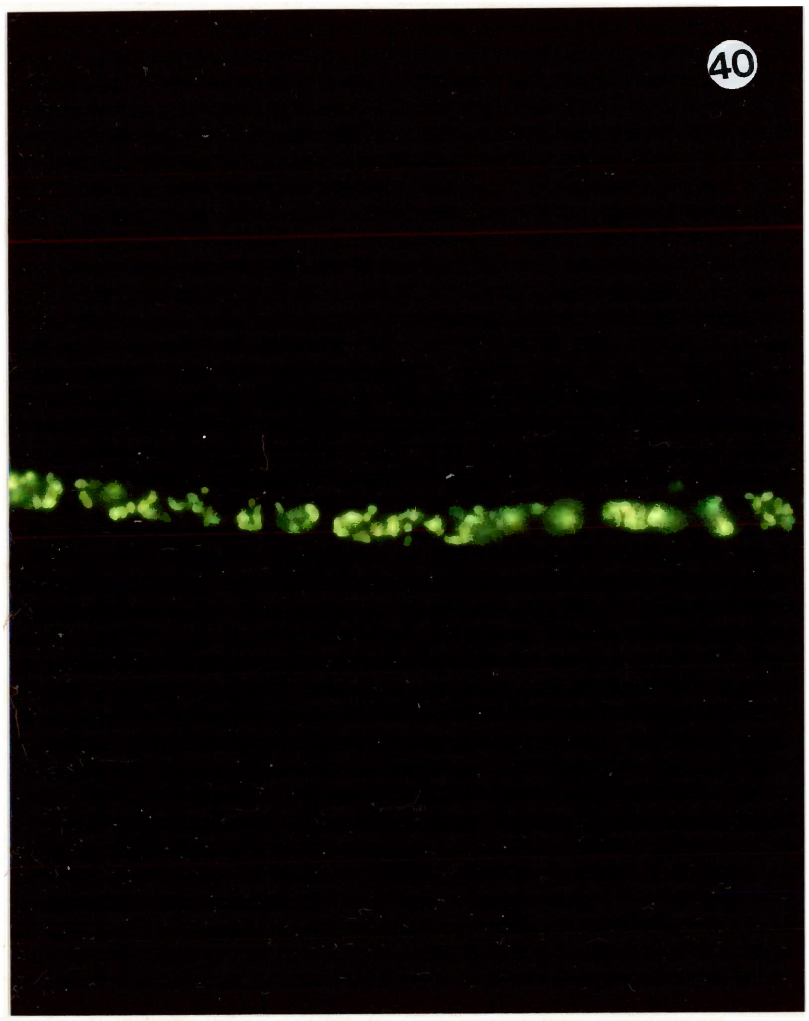
Figure 40. Yellow particles in the cytoplasm of DAPI stained hyphal cell of *F.velutipes* isolate Malloch 8. 12. 73/8 (Mag. X 4 800).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SCANNING ELECTRON MICROSCOPY

Basidia in the hymenium of isolate 188690 were predominently four sterigmate (Figs. 41 and 42; Table 14). However, variations in number of spores were observed in both the basidiocarps of isolate 188690 (Table 14). Some basidia were three sterigmate (Fig. 44) while rarely some were five sterigmate (Fig. 53, 54 and 55). Different stages of basidial development were observed in both three and four spored basidia (Fig. 43, 44, 45 and 46; Fig. 47, 48, 49 and 50). Basidiospores were equal in size for basidia with four spores and unequal in size for basidia with less or more than four sterigmata (compare Fig. 44 and 50). Sometimes tetrads were clumped together (Fig. 50), or had three spores close together and one free (Fig. 51), or all 4 free (Fig. 52), or all four clumped in one mass. Some of the basidia appeared to have aborted sterigmata (Fig. 46). Basidia at the gill margins were collapsed and so expanded basidia nearest to the margins were scored. Variation in the number of basidiospores/ sterigmata was similar at the center of a sample and at the area near the margins.

Table 14. Spore variation on basidia of *F. velutipes*, isolate 188690.

No. of spores	Basidiocarp	
	A	B
5	1	0
4	89	93
3	8	6
2	2	1
1	0	0
No. of basidia sampled :	100	100
ASN (Average spore no./basidium):	3.89	3.92

Scanning electron microscopy of the hymenial layer of *F.velutipes* isolate 188690.

Figure 41. Side view of hymenial layer.

Figure 42. Top view of hymenium, basidia usually with 4 spores.

Figure 43 - 46 Developmental stages of three spored baidium

Figure 43. Basidium with 3 sterigmata.

Figure 44. Unequal spore size and presence of a globule at the base of a spore (arrow).

Figure 45. Equal sized spores.

Figure 46. Mature basidiospores.

Figure 47 - 52 Developmental stages of four spored basidium.

Figure 47. Basidium with 4 sterigmata.

Figure 48. Initiation of basidiospores as knobs at the apical end of sterigmata.

Scanning electron microscopy of the hymenial layer of *F.velutipes* isolate 188690.

Figure 47- 52 Developmental stages of four spored basidium.

Figure 49. Young basidiospores.

Figure 50. Mature basidiospores in a tetrad.

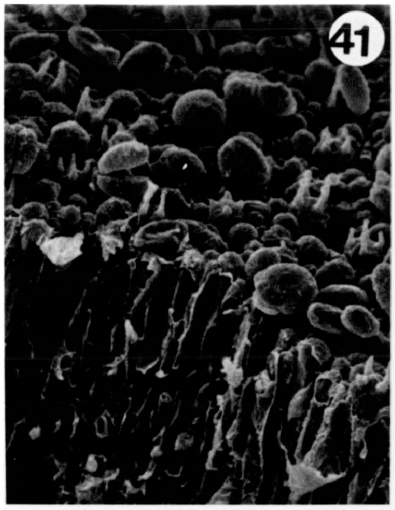
Figure 51. Three of the four spores closely appressed, one free.

Figure 52. Two basidia with difference in spore arrangement. In one of them all four spores are free. In another two are free and two are fused together.

Figure 53. Young five-spored basidium.

Figure 54. Inset of figure 53 showing rare occurrence of a five-spored basidium.

Figure 55. Five mature spores on a basidium.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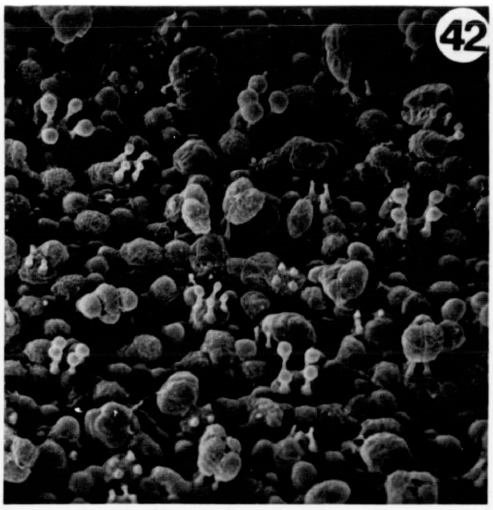
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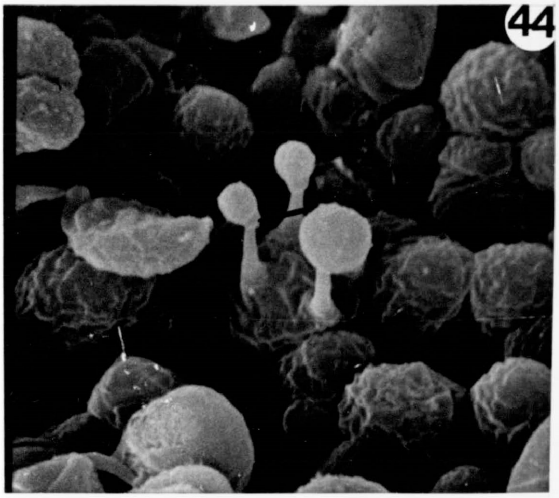
43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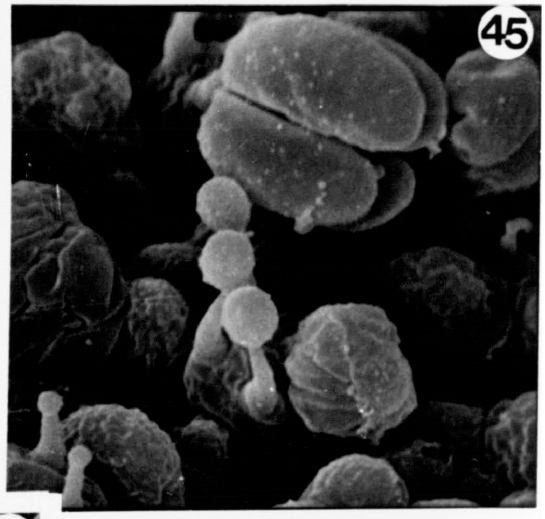
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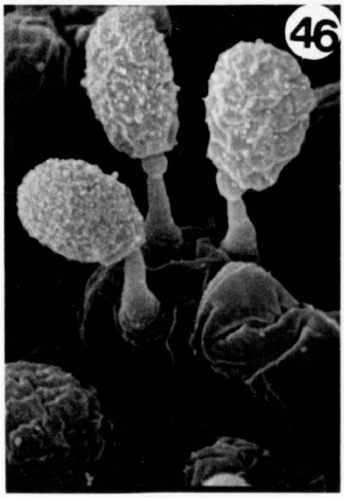
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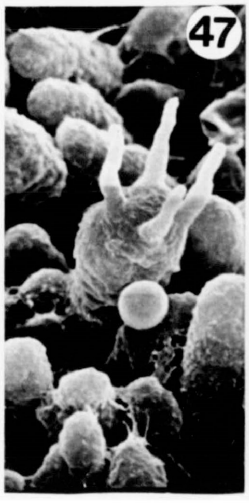
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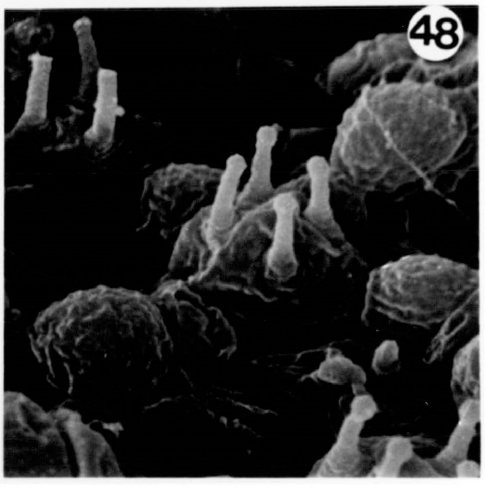
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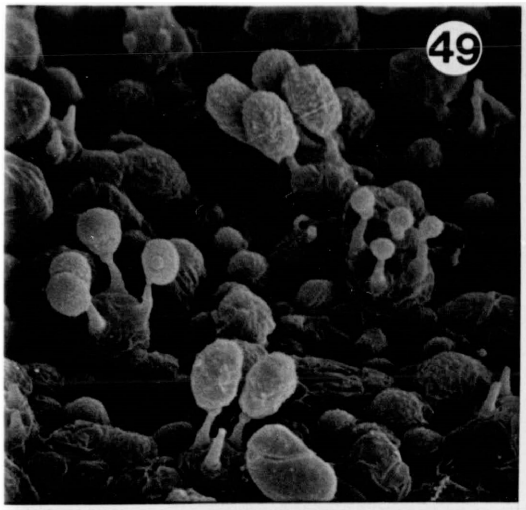
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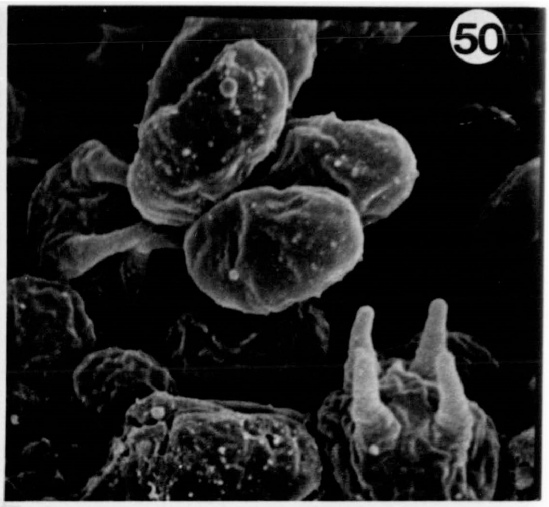
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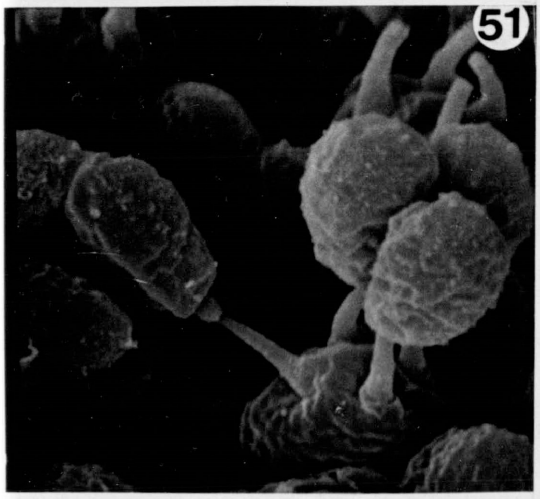
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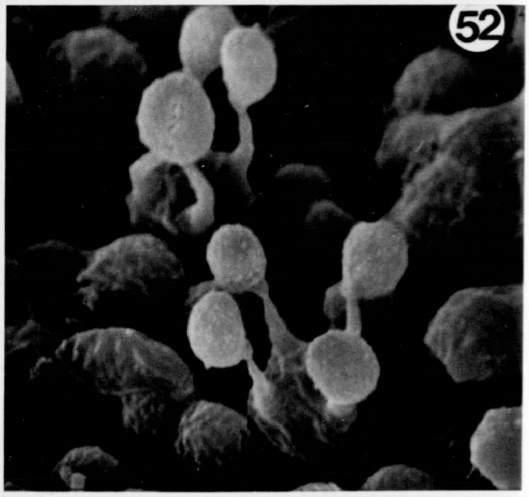
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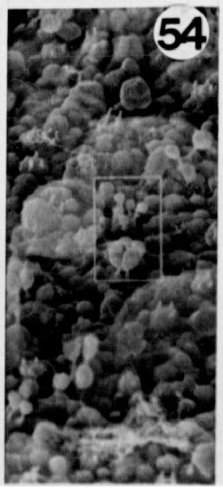
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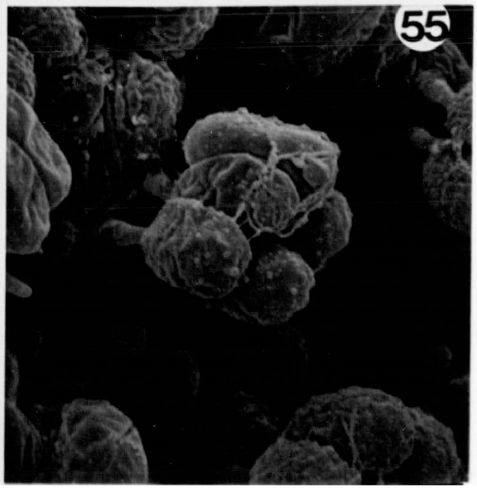
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DISCUSSION

The basic pattern of meiosis in *F. velutipes* is comparable to that found with other basidiomycete species (Mc Claren, 1966; Setliff *et al.*, 1974; Wells, 1977). Karyogamy was followed by two successive divisions of the fusion nucleus that resulted in four, presumably haploid basidiospores. Finally, a post-meiotic mitotic division in the spores resulted in binucleate basidiospores. Basidial development was asynchronous and both first- and second meiotic division occurred at the apical end of a basidium. Spindles were oriented in a transverse to oblique manner. This type of spindle orientation has been reported in *Peniophora quercina* (Kuhner, 1977) and was described as hemichiastobasidial type by Boidin (1958). Elongation of chromosomes during pachytene was succeeded by condensation during diplotene (Figs. 5 and 6). In this respect it differs from *Coprinus lagopus* (Fr.) Fries where chromosomal elongation preceeds karyogamy and chromosomes maintain their size in the following stages of meiosis (Lu, 1970).

Top view of metaphase-anaphase I with "parallel" arrangement of chromosomes around a clear zone observed in the species has been reported for other species (Mc Claren, 1966; Setliff *et al.*, 1974; Wells, 1977). Ultrastructural studies of this stage in *Poria latemarginata* (Dur. & Mont.)Cke. (Setliff *et al.*, 1974) and *Pholiota terrestris* Overholts (Wells, 1977) revealed that continuous fibers were present

between the SPBs and the chromosomes were arranged in "parallel rows" along the continuous microtubules. Anaphase movement involves an increase in distance between the poles and shortening of poles to chromosome distance. Presumably this is accomplished by elongation of continuous microtubules and shortening of chromosomal microtubules (Wells,1977). Different views have been expressed with respect to probable causes of chromosomal movement (Aist and Williams, 1972; Setliff *et al.*, 1974; Wells, 1977). Although the random arrangement of chromosomes at anaphase supports the view that movement towards poles is asynchronous, further work is needed to substantiate the idea.

Abnormalities in nuclear numbers in mycelia of fungi is a common occurrence (Aist and Williams, 1972; Brushaber and Jenkins, 1971). For *F. velutipes* these irregularities in nuclear distributions were observed for both clamped and simple septate isolates. It appears from the greater number of beaked and elongated nuclei as opposed to condensed nuclei, close proximity of anucleate and multinucleate hyphal cells and bridges between the cells that there was a high degree of nuclear migration in the mycelia. The abnormalities in numbers may result from nuclear divisions unaccompanied by septation in a cell or due to unequal distribution of daughter nuclei after anastomoses between two cells (Brushaber and Jenkins, 1971). Formation of pseudoclamps in isolates such as 188690 may also contribute to this distribution, resulting in one less nucleus in the branches arising from such cells (Butler, 1972).

The basic nature of the yellow particles in the cytoplasm of DAPI-stained mycelia is unknown at present; however, they are similar to those seen in studies made of *Rigidosporus vinctus* (pers. comm. Dr. Setliff, April 1987). Of the five isolates, those exhibiting none or few particles, i.e. ECS-1669 and ECS-1673 respectively were collected more recently and were subcultured for shorter

duration as compared to the other three isolates that visually exhibited greater amounts of yellow particles in their cytoplasm. Also, the lack of yellow particles in the controls suggests that they are DNA particles. Cytoplasmic particles in yeast cells have been described as mitochondrial DNA (mt DNA) by Williamson and Fennell (1975) and DAPI positive particles have been observed in HeLa cells deliberately infected with mycoplasmas and vaccina viruses (Russell *et al.*, 1975). It is known that mt DNA can travel within a cell and can be integrated into the nucleus (Wright and Cummings, 1983; Farrelly and Butow, 1983) and in maize plants DNA sequences common to the nucleus, mitochondrial genomes and virus-like elements that help move the mt DNA have been detected (Kemble *et al.*, 1983). Interestingly, virulence of pathogenic bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes* is conferred by plasmids. The T-DNA of plasmid is inserted into the plants and is later incorporated into the host plant nucleus (Chilton *et al.*, 1987). Recently, fungal viruses and extrachromosomal elements in fungi, i.e. DNA plasmids and mt DNA have been shown to be intermediates in the movement of transposable elements around the yeast genome (Buck, 1986). In *Flammulina velutipes*, relatively greater amounts of particles in some cells than the others and few empty cells with particles confined to the walls, suggests that possibly the DNA particles interfere with the development of the fungus and may be responsible for abnormalities in nuclear number and number of spores.

Variations in spore numbers from cultivated material has been linked to refrigeration of the material prior to examination (McLaughlin, 1982; Kerrigan and Ross, 1987). A cold- induced fruiting process of *F. velutipes* perhaps is one of the factors that leads to spore number variation. Similar variations have been observed in nature by Kerrigan and Ross (1987) and they regard the reduction in spore number as a reflection of the temperature of the basidiocarp environment. Basidial collapse at the margins due to loss of turgor and resulting disorientation of the hymenium perhaps contribute to abnormalities

such as aborted sterigma and unequal spore sizes (Kerrigan and Ross, 1987). If the number of nuclei per spore is linked to the reduction in number of sterigmata/spore (Kuhner, 1977), then a relationship between fewer tristerigmatic basidia and rare trinucleate basidiospores is possible. Similar reasoning could be extended to basidia bearing five sterigmata and uninucleate and anucleate spores. Variations in number of spores and in the nuclear content of the spores increases the chances of obtaining heterokaryotic spores in addition to the homokaryotic spores. The heterokaryotic basidiospores coupled with variation in number of spores on a basidium offer great reproductive verstaility to *F. velutipes* and the potential for homothallism. As Kuhner(1977) noted " ...reduction of sterigmata below four is certainly one important cause of amphithallism....", chances of obtaining a heterokaryotic spore are increased when fewer than four spores are initiated on a basidium. However, more strains need to be studied and a more thorough genetic analyses need to be carried out for several strains before the extent of homothallism can be established in the species.

CONCLUSIONS

F. velutipes mycelium exhibited irregularities in nuclear distribution; among strains, heterokaryotic mycelia were differentiated from homokaryotic mycelia on the basis of clamp connections and the prominence of paired nuclei. The species produced predominantly binucleate basidiospores and uninucleate oidia.

There was a marked reduction in the number of nuclei in the hymenium as compared to the stipe or vegetative mycelium. Meiosis in the basidium was fundamentally similar to that of other homobasidiomycetes. Presence of binucleate spores indicated a post-meiotic mitotic division in the basidiospores. Basidiocarps had basidia that varied in number and size of basidiospores, although the basidia usually bore four spores. Future genetic studies must take this variation in spore number and possible occurence of cytoplasmic DNA particles into consideration.

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