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# Breeding And Preliminary Characterization Of Novel Lentinula Edodes (Shiitake) Strains

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# Breeding and Preliminary Characterization of Novel *Lentinula edodes* (Shiitake) Strains

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# Biographical Sketch

Dietrich J. Blum was born in Charlotte, NC in 1973. He attended high school at The Field School and School Without Walls in Washington, D.C., graduating in 1991. After working for several years, primarily in applied fermentation, food service and agriculture he returned to scholastic endeavors attending Warren Wilson College in Swannanoa, NC and University of North Carolina in Asheville. He graduated from Warren Wilson College in 2009 with a B.S. in Biology. While attending Warren Wilson he received a Yarbrough Research Grant and presented original research on the Phytoremediation of Lead by *Hordeum vulgare* to the North Carolina Academy of Science. In the fall of 2010 he entered the Master's program in Plant, Soil, and Environmental Science in the Department of Natural Resources and Environmental Design at the North Carolina Agricultural and Technical State University maintaining a grade point average of 4.0. He is a member of the honor society of Phi Kappa Phi.

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## Abstract

In order to increase productivity and yield of the edible shiitake mushroom, *Lentinula edodes* (Berkley) Pegler, monokaryons from each of six strains, in the culture collection of the Mushroom Biology and Fungal Biotechnology Lab were inter and intra bred to produce 566 novel strains. The objective of this study was to breed and characterize progeny strains for superior mycelial growth and temperature tolerance. Parental strains, monokaryons and their progeny were evaluated for mycelial growth rate at 10-30$^{o}$C. The mating type of monokaryons and dikaryotic progeny was determined. Five of six parents were found to have homozygous mating type alleles indicating limited genetic diversity between these strains. The $B_2$ mating allele was significantly limited ($x^2$ (1) = 41.33, $p < .001$) in monokaryons from four of these parents. A weak relationship was observed between dikaryotic and constituent monokaryotic growth rates at 15-30$^{o}$C ($p < .001$- $p = .0137$, $R^2 = .01 – .02$), leading to the recommendation that monokaryotic growth rate should not be a selection criterion when choosing *L. edodes* monokaryons as breeding stock for growth rate. Strains produced by interbreeding were found to have higher growth rates than those produced by intrabreeding ($p < .0001$) from 15-30$^{o}$C. Strains with significantly faster growth rates ($p < .05$) than both their parents were identified. This research works towards one goal of the mushroom program at NC A&T State University, to develop strains of shiitake targeted for use in different climates, specific seasons, and temperature specific applications in North Carolina and beyond.

# CHAPTER 1

# Introduction

## 1.1 Overview

*Lentinula edodes* (Berkley) Pegler, known commonly as the shiitake mushroom, is a choice edible mushroom with good nutritive value which produces medicinally and biologically active compounds. *L. edodes* is a saprobic white rot fungus decomposing hemicelluloses and lignin along with cellulose (Leatham, 1985; Moyson & Verachtert, 1991; A. Philippoussis & Diamantopoulou, 2011). It is found in the wild on the wood of broadleaved hardwood tree species (D. Pegler, 1983; Stamets, 1993). Wild populations have been reported in China, Japan, Korea, Taiwan, Thailand, Burma, North Borneo, the Philippines, and Papua New Guinea (Arora, 1986; Wasser, 2005).

Cultivation of *L. edodes* fruiting bodies first started in China between 960-1127 BCE (Przybylowicz & Donoghue, 1988). Cultivation began and has continued using hardwood logs as substrate on which to produce mushrooms. More recently, mushroom cultivation has been accomplished using steam sterilized/pasteurized hardwood sawdust and woodchip blocks as well as wheat straw supplemented with cereal grains, bran and/or combinations of many types of lignocellulosic agricultural wastes (A. Philippoussis & Diamantopoulou, 2011; Przybylowicz & Donoghue, 1988). Cultivation of mycelia in liquid media for medicinal and biologically active compounds has been an emerging area of interest as well (Lindequist, Niedermeyer, & Jülich, 2005; Wasser, 2005).

*L. edodes* mushrooms are the second most cultivated species of mushroom worldwide after *Agaricus bisporus,* the white button, brown crimini and portabella mushrooms (S. Chang,

1999; Gold, Cernusca, & Godsey, 2008). The popularity of *L. edodes* is increasing as the economic benefits to cultivators and knowledge of its nutritional and medicinal value become more widely known (Marshall & Nair, 2009). It is necessary to develop novel strains of *L. edodes* in order to continue to have productive strains for traditional cultivation on logs as well as to efficiently exploit newer methods of cultivation on supplemented sawdust/woodchip blocks, agricultural and agroindustrial residues as well as other various biotechnological products. This is especially important knowing that most strains used in industrial production in the US today were imported from Asia.

Development and identification of strains which have high mycelial growth rates at the extremes and across the functional temperature range of *L. edodes* can increase yield and profitability of production operations. Strains with higher growth rates can establish themselves more quickly on a given substrate, outcompete other microorganisms and decrease crop loss due to contamination. Savings of energy and time are realized when incubation time, the longest stage in production, can be reduced. The reduced incubation time shortens the crop cycle and increases annual yield. The reduced time spent in incubation lessens the associated heating and cooling costs of an incubation room. These savings may be increased by selecting strains which grow rapidly at a temperature closer to ambient climatic conditions, reducing the need to raise or lower the temperature in the incubation room and thus reducing the amount of energy used for heating and cooling. These savings increase revenue, decrease overhead and reduce the overall energy footprint of a production system with the associated benefits to the environment.

*L. edodes* has been found to have a bifactorial tetrapolar mating system (Ursula Kües, James, & Heitman, 2011). This system, common to Basidiomycetes, has been researched using the model organisms of *Schizophyllum commune* and *Coprinus cinereus* (Raper, 1966).

Organisms with a tetrapolar mating system have two mating loci referred to as A and B loci or mating types. In order for successful mating to occur gametes must have heterozygous alleles at both mating loci. These A and B loci have been found to be multiallelic and are comprised of subloci which can recombine to form functionally different mating types (L. A. Casselton, 1997; Erika Kothe, 1996). It is estimated that there are 121 A mating types and 151 B mating types in wild *L. edodes* populations in China and 40 A mating types and 63 B mating types in wild *L. edodes* populations in Japan (Li, Xu, Lin, Cheng, & Lin, 2007).

Research has indicated that in China and Japan *L. edodes* strains used in commercial production of fruiting bodies are genetically similar indicating a high level of relatedness (Chiu, Ma, Lin, & Moore, 1996; Terashima, Matsumoto, Hasebe, & Fukumasa-Nakai, 2002; Zhang et al., 2007). This genetic bottleneck must be addressed in order to create a robust population of commercial strains for use in the expanding market of shiitake cultivation. Adding genetic diversity to a breeding program will allow for development of strains targeted for use at temperatures higher and lower than the optimum temperature for *L. edodes* as well as strains suited for use in supplemented sawdust fruiting blocks, solid state fermentation of agricultural byproducts and liquid state fermentation. Adding genetic diversity to the commercial production stock aids the producer in addressing the continuous problem of strain deterioration due to an accumulation of genetic mutations during continuous use and storage (Chakravarty, 2011).

It has been suggested that accumulated deleterious mutations can have an effect on the distribution of mating types among germinated spores (Fox, Burden, Chang, & Peberdy, 1994). A theoretical model of the tetrapolar mating system indicates that the ratio of the mating types of spores will have all four of the possible mating types from a given parent in a 1:1:1:1 ratio. Two of the four mating types are identical to the parents' nuclei and the other two are the product of

recombination during meiosis. The tetrapolar mating system facilitates the successful mating between monokaryotic colonies originating from the spores of parents with different sets of mating types. Successful intrabred matings and interbred matings between monokaryotic cultures from spores originating from parents with the same set of mating type alleles will be limited to a 25% success rate. Successful matings between monokaryotic cultures originating from spores of parents each with unique sets of mating type alleles will be much higher, with up to a 100% success rate (Kendrick, 2000; E. Kothe, 2001; Raper, 1966).

*L. edodes*, as with other Basidiomycetes, can live and grow vegetatively in its gametic stage. The presence of and ability to maintain this vegetative gametic stage *in vitro* has lead to the idea that phenotypic traits of the gametic, more properly monokaryotic, stage are heritable to the dikaryotic fertile stage of development as reported in *Pleurotus sapidus* (S. S. Wang & Anderson, 1972). With regard to growth rate, the extension of this theory is that combining monokaryons with high mycelial growth rates will produce dikaryons with high mycelial growth rates. This theory is not supported in studies with *Lentinus squarrosulus* by O. S. Isikhuemhen, Adenipekun, and Ohimain (2010) or in studies with *Schizophyllum commune* by Simchen and Jinks (1964). Working with *L. edodes*, Miyazaki (2008) found a relationship between monokaryotic growth rates and their dikaryotic progeny in 4 of 5 groups studied but stated that monokaryotic growth rates should not be used as a selection criterion for choosing monokaryons for mating. Determining the validity of this theory for *L. edodes* will be useful in breeding programs to decrease the time and materials spent on selection of monokaryons.

Genetic diversity between different strains of *L. edodes* in commercial and wild populations has been correlated with two different quantifiable traits by comparative analysis with molecular genetics techniques. The first is the phenotype of fruiting temperature optima.

The second is the genotype of mating type alleles. Strains with either different fruiting temperature optima or different mating type alleles have been found to be more distantly related than those with similar fruiting temperature optima or mating type alleles (Fox et al., 1994; Fukuda & Mori, 2003; E. Kothe, 2001).

The traditional temperature based system for classification of *L. edodes* strains uses fruiting temperature optima to classify strains into cool temperature, warm temperature, mid temperature and wide temperature groupings (Chen, 2001). Rather than using the traditional system for classification of *L. edodes* strains a classification system based on the statistical analysis of respective mycelial growth rates on PDA media at 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C is outlined in this work. The proposed system uses mycelial growth rate data, analyzed by ANOVA followed by Duncan's multiple range tests, to construct a growth rate temperature profile. This profile is based on the statistically significant ($p < .05$) differences in mycelial growth rates at 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C.

The primary goals of this research were to breed novel strains of *L. edodes* with high growth rates for use at specific temperatures and across temperatures from 10-30$^{o}$C, lay the foundation of a strain improvement program and increase the number of novel *L. edodes* strains in the Mushroom Biology and Fungal Biotechnology Lab (MBFBL) culture collection.

**1.2 Objectives**

1. Determine mating compatibility, mating type allele frequency and distribution among monokaryotic isolates of six strains of *L. edodes*.

2. Determine the relationship between dikaryotic mycelial growth rate and the mycelial growth rates of their constituent monokaryons for *L. edodes.*

3. Characterize mycelial growth rates of progeny from mating compatibility tests at 10$^{o}$C, 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C.

**1.3 Hypotheses**

Due to the ability to culture gametic monokaryons and keep them *in vitro* the idea of using monokaryotic growth rate as a selection criterion for breeding dikaryons with increased mycelial growth rates is captivating. Mycologists have studied the effect of monokaryotic growth rates on the growth rates of dikaryotic progeny of different species and have found a relationship in some species (S. S. Wang & Anderson, 1972) and a little to no relationship between these growth rates in other species (O. S. Isikhuemhen et al., 2010; Simchen & Jinks, 1964). This relationship has been investigated for *L. edodes* by Miyazaki (2008) with the report that there is a relationship between the mycelial growth rates of dikaryotic progeny and the mycelial growth rates of their constituent monokaryons but that this relationship should not be used as a selection criterion for monokaryons. This finding while clear is available in English only as an abstract. In order to elucidate the findings of Miyazaki (2008), this issue is investigated with the hypothesis that the breeding of monokaryons with faster growth rates will result in progeny with faster growth rates and the breeding of monokaryons with slower growth rates will result in slower growing progeny.

Based on the concept of heterosis, improvement of a given trait by crossbreeding individuals which are not related, and the specific report by Yan and Jiang (2005) of heterosis leading to increased mycelial growth rates in *Stropharia rugoso-annulata* at different incubation temperatures it is hypothesized that novel strains with increased mycelial growth rates will be produced by interbreeding of monokaryons from different *L. edodes* strains. It is hypothesized

that novel strains produced by interstrain crosses will have higher mycelial growth rates than those produced from intrastrain crosses. Based on the same concept, it is hypothesized that strains with mycelial growth rates superior to their parents for each of the temperatures studied and across several of these temperatures will be produced by interstrain breeding.

# CHAPTER 2

## Literature Review

### 2.1 Taxonomy and species description

*Lentinula edodes* (Berkley) Pegler is a choice edible mushroom native to Southeast Asia. Wild populations have been reported in China, Japan, Korea, Taiwan, Thailand, Burma, North Borneo, the Philippines, and Papua New Guinea (Arora, 1986; Wasser, 2005). The current taxonomic position of *L. edodes*, according to Index Fungorum, is shown in Figure 2.1 (indexfungorum.org, 2012). *L. edodes* was moved from the genus *Lentinus* to *Lentinula* based on the hyphal morphology and gill arrangement in 1975 by Pegler, though the genus name *Lentinus* is still used by some commercial growers and field guides. *Lentinula edodes* has monomitic hyphae and more or less parallel gills while members of *Lentinus* have dimitic hyphae and irregular, interwoven gills (D. N. Pegler, 1975; Stamets, 1993). This taxonomic change has been supported by rDNA analysis (Hibbett & Vilgalys, 1993).

*Lentinula edodes* has a dark brown to light brown pileus (cap) which is convex to plane at maturity. The margin is even to irregular, beginning as in-rolled with an under-curved lip, but becoming flattened and irregular as the fruit body ages. Remnants of the partial veil may remain visible along the margin especially when young. The stipe (stem) position is variable. It may be almost lateral, eccentric, or central depending on environmental factors. The stipe is light brown to tan in color and is tougher and more fibrous in texture than the pileus. The gills are white to off white as is the flesh, but both may bruise to a brown color. With regard to cystidia, sterile cells found between spore forming basidia cells, *L. edodes* does not have pleurocystidia, cystidia found on the sides of gills, but does have cheilocystidia, cystidia found on the edge of gills.

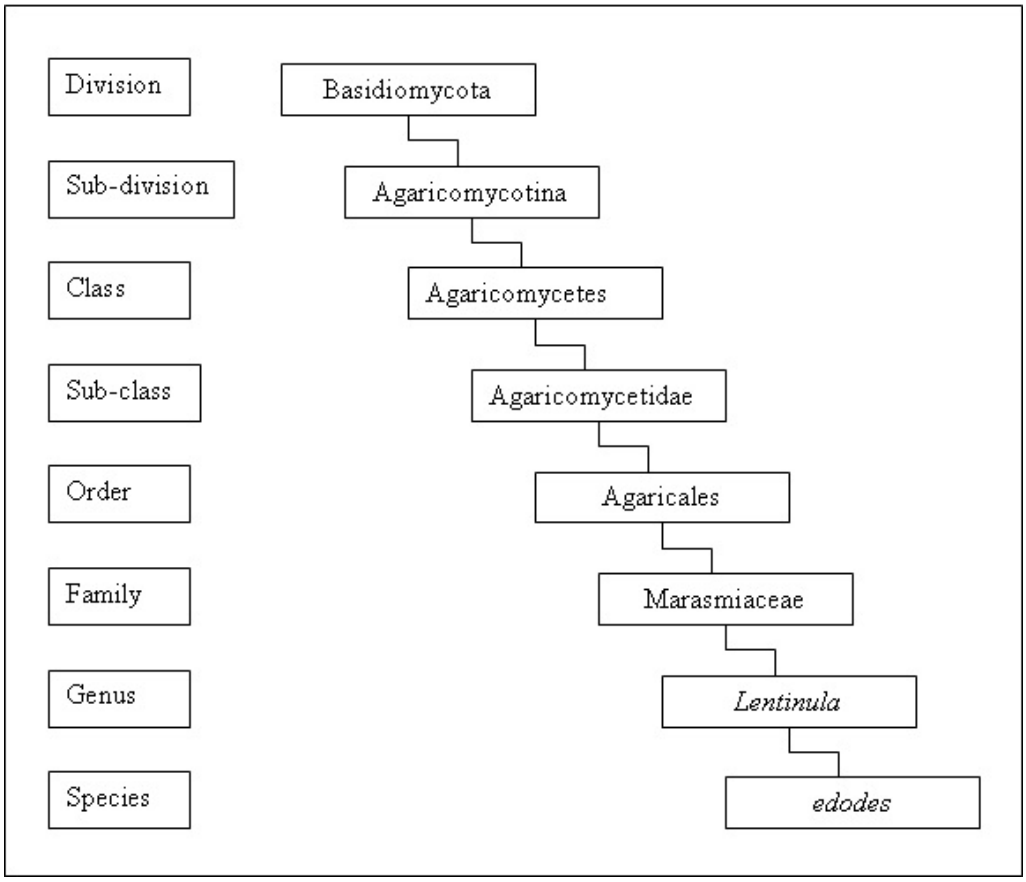



*Figure 2.1.* Taxonomic position of *L. edodes*.

The spores of *L.* edodes are 5-6.5 x 3-3.5 μm, asymmetrically ellipsoid, white to buff in color, smooth with an obvious apical pore. *L. edodes* basidia bear four basidiospores. As mentioned above, hyphae are monomitic and dikaryotic hyphae have clamp connections. Mycelia are white turning tan to brown in older cultures and can be appressed to aerial, sometimes forming hyphal knots (D. Pegler, 1983; Stamets, 1993)

Functionally *L. edodes* is a saprobic white rot fungus, preferentially decomposing hemicelluloses and lignin over cellulose and preferring broadleaved hardwood tree species. The preference for degrading lignin over cellulose leaves the wood bleached in appearance due to the presence of the white color of cellulose and the depletion of dark brown colored lignin, hence the

term white rot. In its native habitat of China, Japan and Korea *L. edodes* is found growing on *Castinopsis cuspidate, Pasania* spp., *Quercus* spp. and *Betula* spp. In these regions *L. edodes* has been cultivated for centuries, where it is commonly called shiitake in Japan and shiang-gu in China (Stamets, 1993).

**2.2 Cultivation and history of *L. edodes***

Cultivation began in China during the Sung Dynasty (960-1127 BCE) and is credited to Wu San Kwung. Wu San Kwung described a method of cultivation including site selection, log choice, and a scoring of the bark with a hatchet, followed by covering the log with soil and keeping the logs moist. After 1 year the soil would be removed and the logs covered with leaves and branches and periodically beaten with clubs to stimulate mushroom formation (Przybylowicz & Donoghue, 1988; Wasser, 2005). These early methods relied on spores from nearby fruiting mushrooms to inoculate the logs. Logs with fruit bodies would be placed near freshly cut logs so the spores would more easily be dispersed to the freshly laid logs (Przybylowicz & Donoghue, 1988).

Modern log cultivation uses a wide variety of hardwoods inoculated with a pure culture of mycelia made into spawn. The fungus is encouraged to colonize the log and generally becomes productive within 6-12 months. After a suitable incubation period these logs can be stimulated to produce sporocarps (mushrooms) by manipulation of their environmental conditions. This is commonly accomplished by soaking the logs in water or otherwise producing a change in humidity, temperature, or a combination of both. Some farmers beat the logs with sticks, mallets or shock them with electricity to stimulate fruit body production. Alternatively the logs may be left in a suitable shady location and when ambient weather conditions are favorable

fruit body production will occur. In temperate climates production is typically heaviest in the spring and the fall (Przybylowicz & Donoghue, 1988).

Log cultivation has been shown to provide a modest supplemental income for some with ready access to hardwood logs, but there are challenges to the outdoor cultivation business model. Log production is often seasonal with gaps in production during the coldest and/or hottest months. Market gluts driving down price can occur as the logs of other growers produce mushrooms synchronously. Another disadvantage to log cultivation is that it requires a large amount of labor. The grower is working with dense, wet, hardwood logs. There is hard work involved in felling trees, bucking logs, transportation, inoculation and setting the log yard. Additionally, there is a lag between the initial investment and revenue as log production requires a 6-12 month incubation period before any return is seen (Gold et al., 2008).

Other modern cultivation techniques use sterilized sawdust based substrate with various combinations of cereal grain, bran, calcium sulfate (gypsum), and/or other lignocellulosic materials to make supplemented sawdust fruiting blocks sometimes called synthetic logs or simply fruiting blocks. Supplemented sawdust fruiting blocks are prepared by mixing sawdust, woodchips, grain, bran, and gypsum. Water is added until the mixture is 60-75% water on a weight/weight basis. This mixture is then packaged into polypropylene bags in preparation for sterilization or pasteurization. Once processed in this way, the fruiting blocks are allowed to cool and are then inoculated with spawn (grain, sawdust or a combination of both and gypsum which has been colonized by a pure culture of the intended fungus). After inoculation, the fungal mycelia are allowed to colonize the fruiting block during an incubation period, in the dark at approximately 21-27$^{o}$C (Stamets, 1993). Towards the end of incubation the outside of the fruiting block becomes covered with white mycelia and takes on a bumpy appearance. This stage

is commonly referred to as the blister or popcorn stage. The initial stage of fruit body formation begins with the formation of aggregations of hyphae called hyphal knots. These knots have the potential to become primordia, which later develop into fruit bodies (U. Kües & Liu, 2000). Indoor production on sawdust based substrate has been shown to provide higher annual revenues than log cultivation. There is very little lag time between inoculation and harvest when compared to log production. Indoor cultivation with consistent, year-round, harvests lends itself to the demands of many larger produce distributers, retail stores and restaurants. Production may be standardized in order to meet demand and attain year-round availability. The disadvantages to producing *L. edodes* on sawdust blocks are the higher associated startup costs, a greater ecological footprint in the form of energy/fossil fuel consumption and often the quality of the finished product. These issues may be mitigated by the shortened time between inoculation and harvest, the use of waste products such as hardwood sawdust, wood chips and agricultural residues followed by good management practices. Strain improvement and development strains targeted for indoor sawdust block production and seasonal/regional temperatures offer further solutions for improving profitability, sustainability quality and of *L. edodes* grown using sawdust block production systems.

**2.3 Market share**

*L. edodes* is one of the five most widely cultivated mushrooms in the world (Wasser, 2005). *L. edodes* mushrooms are the second most cultivated species of mushroom worldwide and in America after *Agaricus bisporus*, the white button and portabella mushroom, as reported by S. Chang (1999) and has remained in that market position as reported in by Gold et al. (2008). China and Japan are the primary producers of shiitake in the world market place. Popularity of *L. edodes* is increasing as its superior taste, texture and knowledge of its nutritional and medicinal

value become more widely known. Establishment of a diverse stock of *L. edodes* for use in America would help farmers increase production and become better able to serve local and world markets.

### 2.4 Nutritive value and medicinal compounds

In general on a dry weight basis *L. edodes* mushrooms contain 58-60% carbohydrates, 20-23% protein (80-87% of which is digestible), 9-10% fiber, 3-4% lipids, and 4-5% ash. *L. edodes* is a source of vitamin D, B vitamins and minerals Fe, Mn, K, Ca, Mg, Cd, Cu, P, and Zn (Wasser, 2005). Medicinally *L. edodes* is the source of lentinan and other water soluble polysaccharides with anti-tumor/anti-cancer properties. *L. edodes* has also been found to reduce cholesterol, lower blood pressure, and boost the effectiveness of the immune system, including fighting viral infections (Rogers & Wasser, 2012; Stamets, 1993; Wasser, 2005). Nutraceutical supplements are produced from mycelium as well as the fruiting bodies of *L. edodes* (Wasser, 2005). Development and identification of strains which have high mycelial growth rates as well as high concentrations of nutritive and medicinally active compounds would be valuable in the production of these products.

### 2.5 Life cycle of *L. edodes*

In order to breed an organism one must understand its life cycle. Many basidiomycetes, including *L. edodes*, can exist as either monokaryotic gametic colonies or dikaryotic colonies at different points in their life cycle. A generalized version of a basidiomycete life cycle follows and is schematically illustrated in Figure 2.2. A basidiospore germinates resulting in monokaryotic hyphae. These hyphae grow and form a monokaryotic colony. The colony can live in this state for an extended period of time and will do so as long as biological requirements are

met. When two monokaryotic colonies of the same species encounter each other the reaction will be based on the mating type of the two individual monokaryons. If their mating type alleles are heterozygous, they will be compatible, if they are homozygous, the organisms will be incompatible. This is known as a heterothallic mating system. Cells of compatible monokaryons will fuse with each other forming a dikaryotic colony. This cellular fusion is called plasmogamy. After plasmogamy the cells are in an n + n arrangement with two unfused haploid nuclei from the participating monokaryotic hyphae present in each hyphal cell.

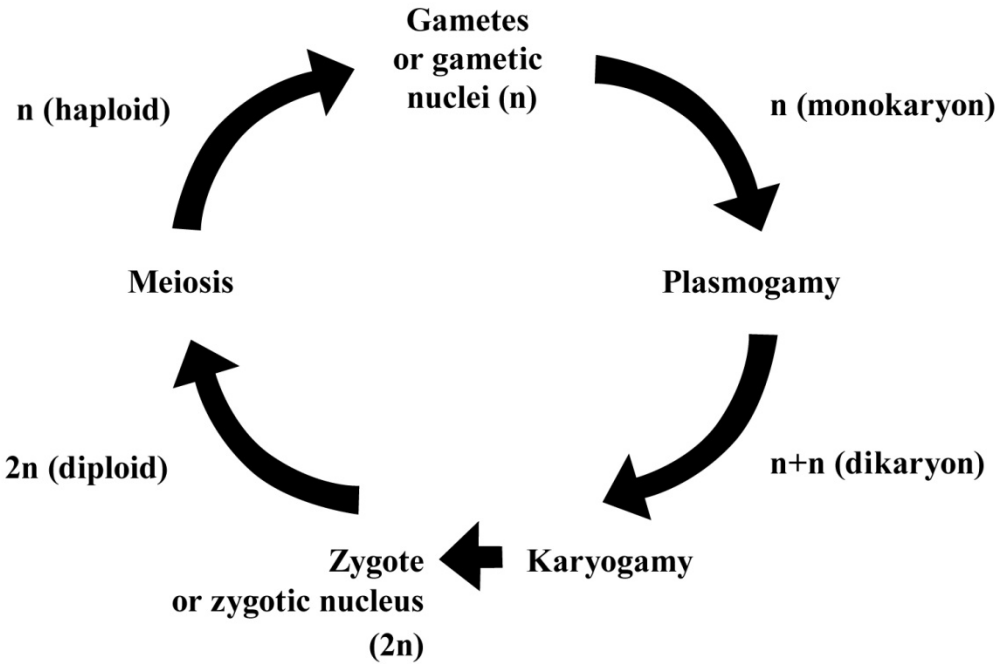



*Figure 2.2.* Generalized nuclear life cycle of Basidiomycetes.

An important and notable morphological feature of dikaryotic colonies is the clamp connection. Clamp connections segregate the two haploid nuclei during mitosis and cell division insuring that one copy of each of the two nuclei is in the each cell. Clamp connections can be

viewed microscopically and are an observable indication of dikaryotization. A schematic diagram showing the function of clamp connections during mitosis is shown in Figure 2.3. A dikaryotic colony is fertile and is able to form a sporocarp, although the timing of the formation of the sporocarp is often a triggered response to environmental conditions. The layer of tissue on the sporocarp, called the hymenium, is where karyogamy quickly followed by meiosis both occur during the preliminary stages of spore production. Cells in the hymenium produce unique terminal hyphae, called basidia, which are the specific location of meiotic events. Meiosis results in haploid spores which are dispersed and begin the cycle anew. (Brown & Casselton, 2001; L. Casselton & Challen, 2006; Raper, 1966).

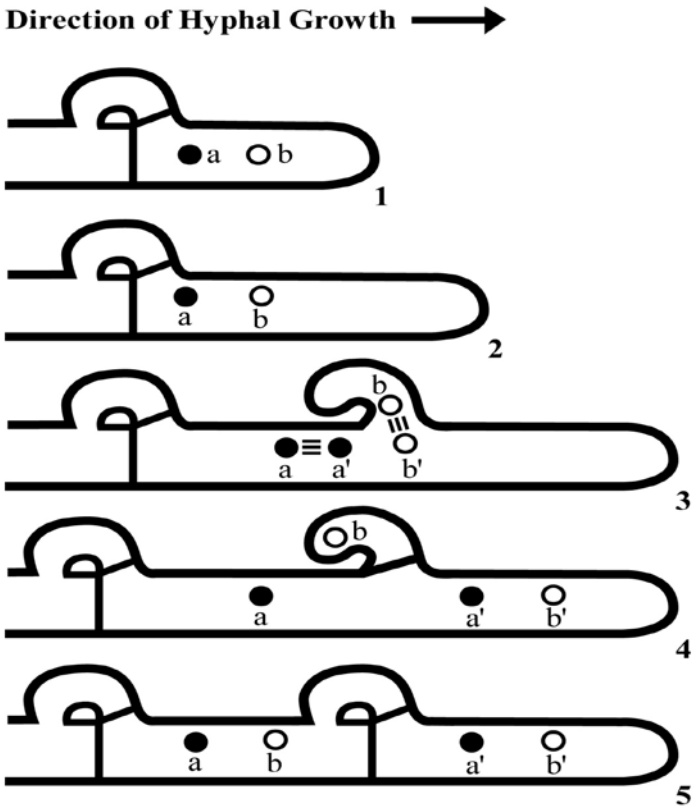



*Figure 2.3.* Schematic diagram of Basidiomycete clamp connection formation, hyphal extension and mitosis progressing in stages 1-5.

The generalized life cycle holds true for *L. edodes*, however the specifics of mating compatibility must be explained in more detail. Mating in heterothallic basidiomycetes, like *L. edodes*, has historically been broken into two categories based on the existence of one or two mating loci synonymously referred to as incompatibility loci. If there is only one mating locus then the organism is referred to as bipolar. If there are two loci then the organism is known as tetrapolar. The single locus in bipolar organisms is labeled the A locus and the two loci in tetrapolar organisms are respectively labeled the A and B loci. The specific alleles which comprise the loci are typically assigned numerical designations. This nomenclature system results in mating types with an A and B each followed by a number e.g. $A_1$ $B_1$. A closer look at this system finds that the A and B loci are multiallelic and are comprised of subloci which can recombine to form functionally different mating types (L. A. Casselton, 1997; Erika Kothe, 1996). Some species have this bipartite structure for both the A and B loci and in some species it is only the B loci that has been found to be subdivided in this manner (James, Liou, & Vilgalys, 2004; L. M. Larraya et al., 2001). This system of subloci and their recombination is the rationale behind the observed abundance of mating type alleles (James et al., 2004). These subunits readily recombine to form new mating types (Cheng & Lin, 2008; Fox et al., 1994; James et al., 2004).

The number of functional mating types is unique to each species of heterothallic fungi. Estimates of the percentage of homobasidiomycetes species with tetrapolar mating systems are 49-65%. Those with bipolar mating systems are estimated at 25% and those with homothallic mating systems are 10-15% (L. Casselton & Challen, 2006). *L. edodes* is a heterothallic tetrapolar basidiomycete. It has been estimated that there are 121 A mating types and 151 B

mating types in *L. edodes* populations in China, and 40 A mating types and 63 B mating types in *L. edodes* populations in Japan (Li et al., 2007).

The tetrapolar mating system allows for up to 100% successful mating between of gametes from non-related individuals of the same species, those with a different set of A and B mating alleles, but positive mating is reduced to 25% between sibling strains and those of the same species with common A and B alleles (Kendrick, 2000; E. Kothe, 2001; Raper, 1966; Stamets, 1993). The high outcrossing rate and low inbreeding rate ensure that natural populations will more often than not outcross, adding to genetic diversity within a population. This may be advantageous in wild populations but can cause breeders trouble by making backcrossing to conserve a desired phenotype time consuming and laborious (E. Kothe, 2001). Identification of mating types produced by given strains and identification by way of uniform tester strains speeds up the backcrossing process (S. T. Chang, Buswell, & Miles, 1993). Conversely outcrossing may provide added vigor in the resulting hybrid strains when compared to the parents and is another laudable goal of breeding programs. Mating type identification can be used as a simple method for evaluating the relatedness of strains which may be used in a breeding program (Fox et al., 1994; E. Kothe, 2001). There are two reasons why unique mating types may be observed between strains: 1. there are high numbers of mating types in the species being studied, 2. the strains are more distantly related (O. S. Isikhuemhen, Moncalvo, Nerud, & Vilgalys, 2000; Raper, 1966).

*Coprinus cinereus* and *Schizophyllum commune* have been used as the primary model organisms to study the bifactorial tetrapolar mating system of heterothallic homobasidiomycetes (Raper, 1966). Study of these organisms has determined that the A and B loci control different physiological responses during plasmogamy of monokaryotic mycelia. For these two model

organisms it has been determined that the A and B loci have unique concerted roles which work by triggering downstream signaling cascades (E. Kothe, 2001; Raudaskoski & Kothe, 2010). The A mating locus codes for transcription factors which control clamp connection formation, synchronized nuclear division, and possibly septa dissolution and formation. The B mating locus codes for pheromones and pheromone receptors which control plasmogamy, nuclear migration, and fusing of the clamp to the cell (E. Kothe, 2001; Raudaskoski & Kothe, 2010). In addition to clamp formation, it has been found that the B mating genes are responsible for the formation of a subapical peg which protrudes behind the septa of the apical cell and rises to meet and fuse with the clamp. Peg formation has been observed in *L. edodes* as well as other basidiomycetes (Badalyan, Polak, Hermann, Aebi, & Kües, 2004; L. Casselton & Challen, 2006).

The A locus has been found to have linked subloci which are redundant with regard to their function in the model organisms of *C. cinereus* and *S. commune* (E. Kothe, Gola, & Wendland, 2003). The subloci are capable of activating common signaling pathways (E. Kothe, 1996). The B locus is also composed of multiple subloci with redundancy built in by way of the pheromones which can activate multiple receptors and receptors which may be triggered by multiple pheromones though not by one produced by subloci on the same primary locus (L. A. Casselton, 1997; E. Kothe et al., 2003). It is not known what specific proteins and peptides are involved in this system or how large the categories of active proteins and peptides are (L. Casselton & Challen, 2006).

A third model organism for the bifactorial tetrapolar mating system is *Ustilago maydis*, the corn smut. When the mating system for *U. maydis* was originally described, the mating loci were labeled in the opposite manner as *C. cinereus* and *S. commune* with regard to their roles in the mating process. With *U. maydis* the A mating locus codes for pheromones and pheromone

receptors and the B mating factor codes for transcription factors (Brown & Casselton, 2001; L. Casselton & Challen, 2006; L. M. Larraya et al., 2001). This is an unfortunate historical situation and while originally somewhat arbitrary convention now favors the labeling of the A and B mating loci using the system exemplified by *C. cinereus* and *S. commune*. Though the specifics have not been worked out for most of the species with heterothallic tetrapolar mating systems the general model is accepted. Molecular sequencing has been used to identify a pheromone receptor gene on the B locus of *L.* edodes suggesting that the *C. cinereus* and *S. commune* model is applicable to this species (Li et al., 2007).

For this work mycelial fusion and the presence of abundant clamp connections is used as an indicator of a positive mating between two monokaryotic cultures with heterozygous mating types at both mating loci. Lack of mycelial fusion and the absence of abundant clamp connections is used as an indicator that the monokaryotic strains being crossed have at least one mating type allele in common.

**2.6 Spore germination**

Spore germination and pairing of monokaryons, although an old technique for breeding is still an effective method of creating hybrid strains (Chakravarty, 2011). Protoplast fusion is also used but is more time consuming and requires greater technical expertise and resources (Chakravarty, 2011). Spore isolation as outlined by Cheng and Lin (2008) is accomplished by suspending discharged spores in sterile water and using a serial dilution to get to a concentration where single *L. edodes* spores can be isolated from a agar based media plate. These cultures are then confirmed to be monokaryotic cultures by the absence of clamp connections using microscopic examination (O. S. Isikhuemhen et al., 2010).

## 2.7 Breeding approaches

In a recent review article “Trends in mushroom cultivation and breeding” Chakravarty (2011) states the lamentable fact that there is not the body of research in the field of mushroom breeding that there is for other crops. Breeding for higher yield and increased product quality were proposed as the primary goals of breeding research. Yield may be increased by focusing on any of several parameters. The most obvious one is increasing the number of quality fruiting bodies produced. Yield may also be increased by breeding to increase the thickness of cap and the density of the tissue (Chakravarty, 2011). Since mushrooms are sold by weight this effectively increases yield. Decreasing the amount of time in the crop cycle is another effective way to increase yield on a seasonal or annual basis (Chakravarty, 2011). Breeding work in this area shows that initiation of fruit body development without a low temperature incubation period is heritable and likely dominant (Sakai, Saito, Kajiwara, & Shishido, 2004). Elimination of the cold temperature treatment step in the induction process shortens the incubation cycle and decreases cost to the producer while increasing annual yield. Another way the crop cycle can be shortened is by increasing the mycelial growth rate of the cultivated organism. This would shorten the amount of time needed for incubation and thereby increase annual yield.

Dovetailing with the concept of decreasing incubation time is the idea of using incubation temperatures closer to the ambient seasonal air temperature. This does not directly increase yield in terms of mass but it can decrease the producers energy costs associated with either heating or cooling the incubation area. This reduction in energy usage translates to an increased monetary yield for the producer as well as a general beneficial environmental impact due to reduced energy consumption. In order to realize these benefits, strains which exhibit high mycelial

growth rates at temperatures above and below the typical optimal temperature must be obtained through breeding and selection.

Other than yield the area with greatest need for strain improvement is resistance to disease (Chakravarty, 2011). Resistance to contamination by *Trichoderma* spp. has specifically been noted as an important problem to address. Outbreaks can quickly infest and severely reduce yield in a commercial mushroom operation. The heritability of direct resistance to *Trichoderma* spp. has been studied (Lee, Bak, Lee, Park, & Ka, 2008). Resistance was measured by placing *Lentinula edodes* and *Trichoderma* spp. in co-culture on PDA and observing the interaction. Some *L. edodes* strains showed the ability to form a barrage, while others either overtook the *Trichoderma* sp. or were overtaken by the *Trichoderma* sp. It was found that direct resistance to this *Trichoderma* sp. was not passed down from parental strains to their offspring. Screening for direct resistance to a contaminant must therefore be done on a strain by strain basis. Breeding studies working with *L. edodes* of know lineage coupled with molecular genetics techniques could be useful in the identification of the resistance mechanism to *Trichoderma* spp. Identification of rapidly colonizing *L. edodes* strains would also be useful in addressing this and other contamination problems since rapid substrate colonization is part of an organism's ability to outcompete undesired contaminating organisms.

The ability to outcompete another organism for the resources of a given substrate is a composite characteristic and makes up the organism's competitive saprobic ability (Shearer, 1995). Part of the mix of traits that enter into the competition equation is the direct resistance to the invading organism, e.g. production of antimicrobial compounds, another factor is the ability to efficiently use the nutrients in the substrate and a third factor is the ability to grow rapidly and colonize the substrate. While these three characteristics are intertwined rapid growth rate can be

used as an assessment of two of these constituent parts of an organism's competitive saprobic ability. A *L. edodes* strain that efficiently uses soluble nutrients and grows rapidly leaves less of a foothold for the pathogen in the substrate (Mata & Savoie, 1998, 2005; Shearer, 1995).

**2.8 Mycelial growth rate on PDA and lignolytic substrates**

As previously noted there are many characteristics which may be selected for through a breeding program. Quantity of sporocarps, size of sporocarp, ability to efficiently utilize a specific substrate, nutritional value, taste, color, concentration of secondary metabolites, and rate of substrate colonization at various temperatures are some of the many traits which may be worthy choices for the focus of a breeding program (S. T. Chang, Buswell, & Chiu, 1993). Mycelial growth rate is a quantifiable trait for which it is relatively easy to generate data. This data is faster to obtain than data for a trait such as yield since one need not produce sporocarps. Though mycelial growth rate may or may not be a trait that producers are interested in directly it could be an indirect measurement of other sought after qualities. High mycelial growth rates shorten the time needed for substrate colonization. This lowers the likelihood that undesirable contaminants, fungi or bacteria, will have a chance to take hold in the substrate and utilize a vacant niche. As previously stated, high growth rates shorten incubation time which shortens the crop cycle and has the effect of increasing annual yield and revenue. Work in this thesis uses the mycelial growth rates of *L. edodes* on PDA as the quality by which strain improvement is determined.

While PDA is not a substrate which is likely to be used as a production media it was selected for use in this work due to its uniformity and availability. When a defined medium, such as PDA is used to evaluate growth rate there are less sources of confounding error than there are

in other media types. There is less variation in the substrate composition than there is in sawdust blocks, logs, or agricultural waste products. While these substrates may be the ultimate intended substrate for production, PDA can be used as a stand in to generate useful data that can be extrapolated to the commercial production potential of *L. edodes*. This line of reasoning is supported by previous work outlined below which uses defined media as a substrate in order to extrapolate information about the performance of *L. edodes* in an *in situ* situation (Mata, Delpech, & Savoie, 2001; Miyazaki, 2008; Puri, 2012; Ryu, Bak, Koo, & Lee, 2009; Tan & Moore, 1992; Tokimoto, Fukuda, Matsumoto, & Fukumasa-Nakai, 1998). Further, mycelial growth rate has been used as an indicator of vigor and ultimate productivity both on defined media and on lignocellulosic substrates (Ashrafuzzaman, Kanruzzaman, Ismail, & Shahidullah, 2009; Curvetto, Figlas, & Delmastro, 2002; Furlan et al., 1997; Levanon, Rothschild, Danai, & Masaphy, 1993; Martínez-Guerrero et al., 2012; Mata et al., 2001; A. Philippoussis, Diamantopoulou, & Zervakis, 2002; Puri, 2011; Tan & Chang, 1989; Tan & Moore, 1992).

A positive relationship between yield and mycelial growth rate on PDA was found in *L. edodes* breeding work using protoplasts (Tokimoto et al., 1998). This work compared the yield of 18 dikaryotic strains grown in log culture to their growth rate on PDA and found the positive relationship to be significant at the 1% level.

PDA was used as the media choice to evaluate the mycelial growth rate of 19 hybrid *L. edodes* strains at low, mid and high temperatures in a dikaryotic-monokaryotic cross breeding program (Ryu et al., 2009). Mycelial growth rate on PDA of hybrid *L. edodes* strains was used to produce quantifiable trait data in order to evaluate the heritability of monokaryotic growth rates to their dikaryotic progeny (Miyazaki, 2008). Tan and Moore (1992) used rapid growth on PDA

media as a selection criterion for 18 *L. edodes* strains which were then grown in supplemented sawdust blocks (Tan & Moore, 1992).

In a study using two strains of *L. edodes* which were grown on five defined agar based media types, Puri (2012) found that PDA was the defined agar media which produced the highest growth rate in both strains. Similar to the work in this thesis, these strains were grown at 15, 20, 25, 30 and 35$^{o}$C. Strain L1 grew at a faster rate than strain L2 at all temperatures on PDA. Strain L1 was found to have a higher yield of fruiting bodies when compared to L2 on 15 different substrate types made from agricultural waste. This supports the concept that faster growth rates are equated with higher fruit body yields and that mycelial growth rate on PDA can be used as a predictor of *L. edodes* yield on agricultural waste.

Mata et al. (2001) determined the mycelial growth rate of *L. edodes* on three agar media types; malt extract agar, malt/yeast extract agar, and malt/yeast/wheat straw extract agar. Mycelial growth rates on all three media types were all found to be positively correlated with *L. edodes* mycelial growth rate on wheat straw. This like the work of Puri (2012) supports the use of mycelial growth rates on agar based media to select *L. edodes* strains for use on lignolytic substrates.

In other work looking at use of wheat straw as a potential substrate for *L. edodes*, rapid colonization was linked to the ability of *L. edodes* strains to use soluble carbon recourses. While the ability to solubilize these resources is one important issue the ability to rapidly metabolize low molecular weight carbon resources is another. The ability to rapidly utilize carbon and other nutrients renders these resources unavailable to potential contaminating microorganisms which resist sterilization/pasteurization and remain viable in the substrate (Mata & Savoie, 1998). Since

defined media like PDA has an abundance of lower molecular weight carbon nutrients it is a suitable media for determination of the relative potential of an organism to metabolize soluble carbon recourses in other more complex substrates. This potential is measured as mycelial growth rate. This does not necessarily predict the relative suitability for use on a complex lignolytic substrate but it does predict how well a given strain can use available resources before competing organisms which may still be present are able to use it. The work of Mata and Savoie (1998) found that the strains with high metabolic activities were also the strains which were first to produce mushrooms and had higher mushroom yields.

Much research has been conducted growing *L. edodes* directly on various lignolytic substrates. The trends in this research show that the faster the mycelial growth on these substrates the lower the rate of contamination and that strains with the fastest growth rates on a given substrate often produce mushrooms more quickly and in greater abundance than those with slower mycelial growth rates. Some of these studies have used multiple strains of *L. edodes* on a limited number of substrates while others have used a limited number of strains to test a wider variety of substrates (Ashrafuzzaman, Kamruzzaman, Ismail, Shahidullah, & Fakir, 2009; Levanon et al., 1993; A. Philippoussis & Diamantopoulou, 2011; A. Philippoussis et al., 2002; A. N. Philippoussis, Diamantopoulou, & Zervakis, 2003; Puri, 2012).

In work examining 12 various substrates produced from the wood of tropical trees and one *L. edodes* strain, it was found that among the substrates tested, mycelial growth was fastest on Jackfruit sawdust. Jackfruit sawdust was first to fruit and had the greatest yield compared to the other tropical wood types tested (Ashrafuzzaman, Kamruzzaman, et al., 2009; Ashrafuzzaman, Kanruzzaman, et al., 2009). This same trend is seen in the work conducted by Curvetto et al. (2002) who studied sunflower seed hulls as a potential substrate for *L. edodes*.

The substrate producing highest mycelial growth rate, containing an 8:2 ratio of sunflower seed hulls to wheat bran, was found to have the highest mushroom yield of those substrates tested. Another study following this trend compared various agricultural wastes for use in the production of *L. edodes* spawn and found that the substrate combinations on which the shiitake strain grew fastest also gave the highest yields when the spawn was subsequently used to inoculate fruiting blocks (Puri, 2011).

While there does seem to be a trend here between mycelial growth rate and yield on these various substrates the literature is not uniform. Zervakis, Philippoussis, Ioannidou, and Diamantopoulou (2001) compared mycelial growth rate and yield of *L. edodes* on wheat straw, cotton gin trash, peanut shells, poplar sawdust, oak sawdust, corn cobs and olive press-cake. A relationship between *L. edodes* mycelial growth rate on wheat straw and mushroom yield was observed, but no relationship was found between the mycelial growth rate and yield for other lignolytic substrates tested. The primary limitations of this work were that Zervakis et al. (2001) only used one strain of *L. edodes* and may have induced fruiting too early since part of their aim was to shorten the time between inoculation and fruit body production. Premature fruiting could have decreased the yields on substrates with higher lignin content. Differences between strains, substrates, incubation time and yield have been observed in work comparing these factors (Royse & Bahler, 1986).

The findings of Zervakis et al. (2001) are in agreement with earlier work conducted by Tan and Chang (1989). Their work compared mycelial growth rate on supplemented sawdust fruiting blocks of various composition to fruit body yields from those substrates. It was concluded that the substrate/strain combinations that had high yields all had high mycelial growth rates, but that not all combinations with high mycelial growth rates had high yields.

In several other studies originating at the National Agricultural Research Foundation in Athens, Greece, as well as the study by Zervakis et al. (2001) mentioned previously, it has been pointed out repeatedly that a rapid mycelial growth rate is important to lessen the risk of contamination when growing *L. edodes* on various agricultural waste products. Additionally, these studies assert that faster growth rates reduce the duration of the incubation phase of mushroom production. These studies use 2-5strains of *L. edodes* and substrates including wheat straw, cotton waste, peanut shells, oak sawdust, reed grass, corncobs and/or bean stalks (A. Philippoussis & Diamantopoulou, 2011; A. Philippoussis et al., 2011; A. Philippoussis et al., 2002; A. N. Philippoussis et al., 2003). When using agricultural wastes as substrates rapid colonization of the substrate is critical due to the high loads of contaminating organisms that are likely to be present even after sterilization or pasteurization. A high growth rate gives the selected organism a competitive advantage during the critical initial colonization phase.

### 2.9 Strain deterioration

Strain deterioration is a continuous problem in mushroom cultivation (Chakravarty, 2011). Repeated subculturing and or prolonged storage can result in strain deterioration due to an accumulation of genetic mutations that have occurred during storage and over multiple cell divisions under *in vitro* conditions (Chen, 2001). The longer a strain is in cultivation and the more cell divisions occur the greater the likelihood of the accumulation of mutations which can decrease the yield and quality of the fruiting bodies (Stamets & Chilton, 1983).

### 2.10 Strain diversity and temperature optima

In addition to the problem of strain deterioration it has been pointed out that there is limited genetic variability in the mushroom farmer’s library of cultivated strains (Chakravarty,

2011). The implication of limited genetic variation in commercial stock is that there is a decreased opportunity for producers to access strains which are free of accumulated mutations. Further, this genetic bottle neck could leave the population of commercial *L. edodes* susceptible to disease and pest attack. The assertion that there is limited genetic variability among commercially used *L. edodes* strains is based on molecular genetics research of cultivated and wild type *L. edodes* strains.

Nineteen strains of *L. edodes* used in commercial cultivation in China and three strains which were collected from the wild were characterized by random amplified polymorphic DNA (RAPD) marker profiles to assess the level of relatedness between them (Chiu et al., 1996). Although the commercial strains were reported to have come from distinct origins, different spawn suppliers and strain improvement programs, they were found to be highly related. It was suggested that the cultivated strains may be related to Japanese strains which were introduced and widely used in the 1960's. The three wild type strains were found to have a greater genetic diversity based on analysis of their RAPD profiles. The investigators concluded that wild populations of shiitake are biodiversity reservoirs and that Chinese commercial strains are highly related.

In Japan 15 cultivated strains were characterized using amplified fragment length polymorphism (AFLP) analysis to assess the level of relatedness between the studied strains. It was found that these 15 strains could be divided into two groups which correspond to their fruiting temperature designation so that one group is comprised of high temperature strains plus strains used for cultivation on sawdust blocks and the other group is made up of strains which fruit at low temperatures (Terashima et al., 2002). This suggests that there is low diversity in cultivated strains of *L. edodes* in Japan. It further suggests that either high temperature strains are

better suited to sawdust block production or that strains used for sawdust block production were derived from high temperature strains. This is also suggestive of a genetic basis for fruiting temperature and is likely the origin of the concept that strains which fruit at high temperature are well suited for supplemented sawdust block cultivation.

Similar research was conducted in China with 15 cultivated strains and two wild type strains. This work used inter simple sequence repeat (ISSR) analysis to determine the level of genetic relatedness between the strains studied (Zhang et al., 2007). As in the study by Terashima et al. (2002), it was found that the 17 strains could be divided into two groups supported by ISSR results. It was also found that these two groups matched those made based on optimum temperature for fruit body formation. One group included the high and broad temperature strains while the other included the low and mid temperature strains. Zhang et al. conclude that "genetic diversity is related to temperature". Additionally they are in agreement with Terashima et al. (2002) that, similar to Japan, the genetic diversity of cultivated *L. edodes* strains in China is low.

Along with pointing out the potential genetic bottleneck in commercial *L. edodes* strains these studies reinforce the concept that there is genetic diversity existing between strains from different temperature classifications. Strains are traditionally classified by fruiting temperature in the following categories: low temperature $10^{o}$C, mid temperature 10-$18^{o}$C, high temperature >$20^{o}$C, and wide range 5-$35^{o}$C (Chen, 2001; Hasebe, Ohira, & Arita, 1998; Terashima et al., 2002). The work of researchers like Zhang et al. (2007) and Terashima et al. (2002) support the use of temperature classification as an indicator of relatedness.

Strains which fruit at low to middle temperatures are typically used for outdoor log cultivation. Strains from the high temperature group are said to be more easily induced and are able to fruit under summer conditions. These strains are used for log cultivation as well as indoor supplemented sawdust block cultivation (Chen, 2001; Stamets, 1993). It has been suggested that strains which are able to live at the upper extreme of the high temperature range (> or = 30$^{o}$C) produce greater yields on sawdust blocks (Stamets, 1993). The hierarchy of genetic dominance of optimum temperature for fruiting has been found to be high temperature > medium temperature > low temperature (Hasebe et al., 1998).

This concept is supported by work done by Ryu et al. (2009) in South Korea. In a dikaryotic-monokaryotic cross breeding program strains were evaluated for sawdust block cultivation. Optimal temperature for mycelial growth of both the dikaryotic and monokaryotic cultures was evaluated. Cultures were classified as low, mid or high temperature strains. It was found that crosses of strains classified as mid-temperature with strains classified as high temperature produced novel strains with increased yield. There was no significant increase in the yield of the hybrid strains produced by the combinations of the other temperature based groupings. This work supports the findings of Hasebe et al. (1998) that high temperature strains have a dominant phenotype.

Though the traditional classifications for *L. edodes* are based on optimal fruiting temperatures a relationship between fruiting temperature and optimal temperature for mycelial growth has been identified. Strains of *L. edodes* which grew well at high temperatures also fruited at high temperatures (20-33$^{o}$C) (B. Wang, Tang, Xiong, Jiang, & Xian, 2004). Based on this work, mycelial growth rate at a given temperature can be used as an estimation of optimal fruiting temperature and comparisons between research using the two parameters can be made.

The identification of these genetically different groups, their associated phenotypes, and the hierarchy of dominance suggests that fruiting as a response to temperature and the inducibility of fruiting can be maintained and selected for through breeding regimes. Inbreeding and hybridization of strains known to possess cold temperature optima should maintain cold temperature optima. Outcrossing between strains with warm and high temperature optima or inbreeding between high temperature strains should produce high temperature strains. Further it has been shown that hybrid strains especially those made by mating parents with different optimum temperatures can produce more vigorous growth and have higher productivity in *Pleurotus sapidus* (S. S. Wang & Anderson, 1972) and in *L. edodes* (Ryu et al., 2009). Based on this information the hybridization of strains with different temperature optima, especially mid range crossed with high range, is a sound strategy for the development of new strains with aggressive colonization and high inducibility. These strains would be well suited for use in sawdust based fruiting blocks, as well as having the potential to increase the length of the log cultivation season into the summer and for use in tropical regions (Mata & Savoie, 2005).

The research in this thesis focuses on the initial characterization of the mycelial growth rates of the parental and novel dikaryotic strains, as well at their monokaryotic constituents at a range of temperatures from $10^{o}$-$30^{o}$ C in five degree increments. The optimal temperature for *L. edodes* mycelial growth is variable for different strains but in general is 20-28$^{o}$C (Zervakis et al., 2001). The use of mycelial growth rates across a range of temperatures as opposed to the use of optimal temperature for fruiting is a proposed shift in the assessment of *L. edodes* strains. The logic behind this change is based on the following three points. First, as previously stated, the work of B. Wang et al. (2004) found that the growth rate at high temperatures correlated with fruiting at high temperatures. Therefore rapid growth at a given temperature can be used to

estimate a functional fruiting temperature. This point has not yet been examined under a wide range of conditions and only the abstract of the cited research is available, as is the case with some Asian language journals. This assertion makes intuitive sense but should be looked into more thoroughly. Second, for log cultivation as well as supplemented block cultivation *L. edodes* spends more time incubating in a vegetative growth stage than in a fruiting stage. In biotechnological applications a fruiting stage may never be used as it may be only the mycelia or their extracellular exudate that is of interest. Therefore the vegetative growth stage is the predominant condition and as such should be used to characterize strains. Third there is less variability in the environmental parameters of an organism growing vegetatively on an agar plate in an incubator than there is in a fruiting house. This makes comparison between work conducted by different experimenters and in different laboratories more uniform.

This research will serve to provide base line information about the suitability of these various strains and their gametes for use in breeding and production across the functional temperature range of *L. edodes* using mycelial growth as a parameter rather than fruiting. Strains that are found to have high mycelial growth rates at the extremes of the temperature study and those that have high growth rates across many temperatures are good candidates for further research.

### 2.11 Strain diversity mating type

Identifying mating types and using mating type as genetic marker is a simple method for determining the genetic diversity of a population or a culture collection. The use of mating type as a measure of relatedness is supported by taxonomists and by researchers using molecular techniques. It is suggested by Fox et al. (1994) that mating type diversity can be used to indicate

a level of relatedness within the species *L. edodes* (Fox et al., 1994). This information can be used in breeding programs to identify diverse lineages for hybridization or used for conservation of a phenotype by means of backcrossing. Having identified four distinct lineages and populations of *L. edodes* from Japan to New Zealand, Hibbett and Donoghue (1996) suggested that shiitake breeders use isolates from outside of northeast Asia to bring useful genetic diversity into their breeding programs.

Mating type was found to be useful for characterizing genetic differences in wild strains of *L. edodes* collected in Japan. Eighteen wild type strains were grouped into distinct genets based on mating type. When concurrent analysis of the same 18 strains was conducted using mitochondrial DNA, the strains were grouped into roughly equivalent genetic groupings (Fukuda & Mori, 2003). The relationship between diversity of mating type and diversity determined by molecular techniques has been used with other species of fungi. High rates of mating compatibility were found between distant populations of *Pleurotus tuber-regium* with lower rates observed between strains from populations originating in geographically closer regions (O. S. Isikhuemhen et al., 2000). Higher rates of mating incompatibility indicate lower numbers of unique mating alleles. Analysis of the internally transcribed spacer (ITS) regions provided evidence that corresponded to the rates of mating compatibility for predicting relatedness. The populations which had higher mating incompatibility were more related and had more mating types in common than the populations which had fewer mating types in common and more readily mated.

### 2.12 Growth rate of monokaryons as a basis for selection

The selection and mating of fast growing monokaryons, in order to produce fast growing dikaryons, is intriguing and would provide a useful methodology for monokaryon selection. The literature does not provide conclusive information with regard to the relationship between monokaryotic growth rates and the growth rates of the resulting dikaryons. The relationship has been found to be only partial and may vary by genus and species.

Breeding work crossing *L. edodes* monokaryotic cultures was conducted by Miyazaki (2008) to determine the heritability of monokaryotic growth rate. Fifteen monokaryons from one dikaryotic parent and five monokaryons each from a unique parent were crossed to produce 75 novel dikaryotic strains. The growth rate of the progeny was determined and the progeny were separated into groups based on the five monokaryons, each of which originated from a unique parental strain. A relationship was found in four of the five mating lines between the mycelial growth rate of the monokaryotic parent and the mycelial growth rate of the resulting hybrid progeny. However Miyazaki (2008) concluded that mycelial growth rate of monokaryons should not be used to select gametes for breeding dikaryons.

S. S. Wang and Anderson (1972) found a significant relationship between the mycelial growth rates of dikaryotic cultures of *Pleurotus sapidus* and their constituent monokaryons. Simchen and Jinks (1964) found a limited relationship between the mycelial growth rate of component monokaryons and the resultant dikaryotic cultures in *Schizophyllum commune*. They concluded that most of the variation in the growth rate of dikaryons was not correlated with the variation in the monokaryotic growth rate. Simchen and Jinks (1964) hypothesized that the lack of a significant relationship was due to epigenetic differences between the monokaryotic and dikaryotic stages of development.

In work done by Larraya, Perez et al. (2001) monokaryotic growth rate was linked to the presence of specific mating type loci and subloci in *Pleurotus ostreatus.* This work identified a trend linking the growth rate of the constituent monokaryons to the growth rate of the resultant dikaryons, but no analysis was conducted to determine the significance of this trend. Yan, Jiang, and Cui (2004) found that protoplasts from *Stropharia rugoso-annulata* with an $A_2B_2$ mating type grew faster than those with an $A_1B_1$ mating type. These studies indicate that while the relationship between the growth rate of monokaryons and the dikaryotic cultures produced by mating is not strong there may be a relationship between specific mating types and their growth rate. This does not provide a clear method of selection for producing fast growing dikaryons since one must have both heterozygous mating types to produce a fertile dikaryotic strain. If fast growing monokaryons from different lineages, with different sets of mating alleles, can be indentified and mating type is correlated with growth rate, then these monokaryons could be paired. This may be a workable strategy to employ the relationship between a given mating type and high monokaryotic growth rates resulting in faster dikaryotic strains.

By determining the mycelial growth rates of the gametes and comparing them to the growth rates of the offspring, it is hoped that a methodical approach to selecting monokaryotic breeding stock may be established with the goal of producing novel strains with high mycelial growth at various desired temperatures.

**2.13 Distorted allele ratio and recombinant mating types**

A theoretical model of mating type allele ratios of spores which are the product of meiosis and based on Mendelian principles would have all four of the possible mating types from a given parent in a 1:1:1:1 ratio. A diagram of this model is shown in Figure 2.4. Two of the four

mating types are identical to the parents' nuclei and the other two are the product of recombination during meiosis. The theoretical mating ratio of 1:1:1:1 is not always observed during experimentation. Distorted allele ratios have been found in *L. edodes* (Cheng & Lin, 2008; Fox et al., 1994) and other fungi including *Pleurotus ostreatus* (Kay & Vilgalys, 1992; L. M. Larraya et al., 2001), *Phytophthora infestans* (Judelson, Spielman, & Shattock, 1995) and *Schizophyllum commune* (Raper, 1966).

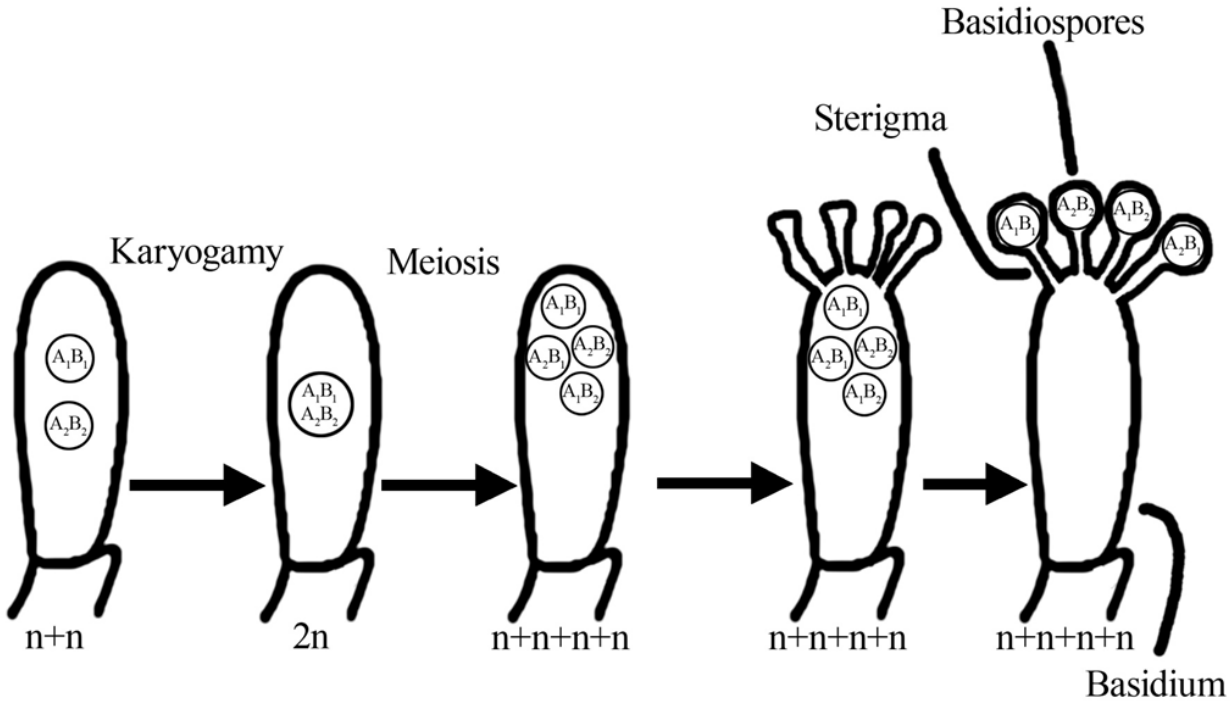



*Figure 2.4.* Tetrapolar model of spore production showing derivation of theoretical mating type allele ratio.

There are several theories as to why distorted mating type ratios occur. It has been shown that wild strains of *L. edodes* have less distortion from the expected ratio than cultivated strains (Cheng & Lin, 2008). It has been theorized that a recessive lethal factor can appear in commercial stock after long periods of storage and repeated subculturing (Fox et al., 1994). An

associated theory suggests that a lethal factor near the mating locus may serve to eliminate gametes with inconsistent mating type allele combinations e.g. those which express both $B_1$ and $B_2$ functions (Judelson et al., 1995). It has also been proposed that rather than a lethal factor there may be a factor causing slow germination or subsequent slow mycelial growth (L. M. Larraya et al., 2001). These factors rather than being strictly lethal would cause a lack of vitality which may in turn cause spores to be under selected during single spore isolation. Thirdly, it has been found that spores with the parental mating types outnumbered those with non-parental mating types (Cheng & Lin, 2008) thus distorting the mating type ratio in the *L. edodes* strains studied. This study found that this distortion was significantly lower in wild type strains than it was in strains from commercial sources. This finding is not inconsistent with the theory that lethal mutations or factors accumulated during storage and or repeated subculturing is the cause of this phenomenon. If this theory holds then the mating type allele ratio could be a useful measure of how much time a strain has been under *in vitro* conditions.

Another type of distortion in the mating type ratio of germinated spores is due to the occurrence of recombinant mating types. Recombinant mating types have been observed in spores from *L. edodes.* These spores were identified because they were able to mate with spores with 2, 3, or 4 different mating types which were generated from the parent (Cheng & Lin, 2008). It was hypothesized that this result was due to mutation or an exchange of genetic material during meiosis or from recombination between mating type subloci. Recombination of mating type alleles of *L. edodes* was observed in the work of Fox et al. (1994). They observed that $B_3$ and $B_6$ mating loci were recombined in the progeny from all four of the strains with those mating types. Recombinant mating type genes have also been found in *Pleurotus djamor* during research into the roles of the A and B mating loci as they relate to the model organisms *C.*

*cinereus* and *S. commune* (James et al., 2004). As mentioned previously, recombinant mating types may be the source of the large number of functionally different mating types and the mechanism for their creation (L. A. Casselton, 1997; Erika Kothe, 1996).

# CHAPTER 3

## Materials and Methods

### 3.1 Fungal strains and isolation of monokaryotic cultures

Six strains of *Lentinula edodes* (Berkley) Pegler from the Mushroom Biology and Fungal Biotechnology Lab (MBFBL) were selected as parent strains. Previous work at MBFBL characterized the parent strains MBFBL 1, 2, 3, 4, 5 and 6 as having superior mycelial growth rates at temperatures of 10$^{o}$C, 10$^{o}$C, 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C respectively (both MBFBL 1 and 2 were selected for 10$^{o}$C) when compared to each other. Spores were collected from the basidiocarps of each parent strain by placing the pileus gill side down on a clean sheet of paper or glass microscope slide and covering with a suitable container until a spore print was produced, usually overnight. Spores from fresh prints or previously collected spore prints which were stored at 4$^{o}$C were used to make spore suspensions in sterile deionized water. A serial dilution of the spore suspension was performed making dilutions from $10^{-1}$ to $10^{-7}$. Nine hundred µl of the spore suspension at each dilution was plated on white rot spore germination media (Table 3.1) in 100mm x 15mm Petri dishes using the spread plate method. Plates were incubated for 7-10 days at 20$^{o}$C and 25$^{o}$C until germination was observed, however plates with dilutions producing few germinated spores were incubated up to 4 weeks. Plates with dilutions that yielded visibly individual germinated spores were used for the isolation of the single spore cultures. From these plates individual spores were removed using a sterilized toothpick to transfer each of the germinated spores and its monokaryotic mycelium to a 60mm x 15mm Petri dish containing Potato Dextrose Agar (PDA) Difco™ media. This process was repeated as needed and used to generate 37 monokaryotic cultures from each of the parent strains. These cultures were examined

at 40x using a light microscope and the squash mount technique. Under these conditions the mycelia were examined for clamp connections. Lack of clamp connections was used as verification that the culture was a monokaryotic single spore isolate.

### 3.2 Media formulations and preparation

PDA media was prepared according to the manufactures specifications by dissolving 39g of Difco™ dehydrated PDA in 1L of deionized water, heating with agitation until the agar was dissolved and autoclaving for 15 minutes at 121$^{o}$C. Modified PDA was prepared in the same way but was supplemented with 2g per L of yeast extract and hardwood extract was substituted for 10% of the deionized water. Hardwood extract was prepared by mixing 100g of dry oak (*Quercus* spp.) sawdust into 1L of deionized water, autoclaving for 15 minutes at 121$^{o}$C and filtering the supernatant. Spore germination media was prepared by adding 1g dextrose, 1g soluble starch, 28g agar, 10ml of hardwood extract and 990ml of deionized water in a 2L flask, followed by heating with agitation until the agar was dissolved. The media was then autoclaved for 15 minutes at 121$^{o}$C. The spore germination medium with 10% hardwood extract was prepared the same way but had 10% hardwood extract added as compared to the 1% used in the first spore germination media described above. Media formulas are shown below in Table 3.1.

Table 3.1

*Media formulas per 1 liter of media*

| | Spore Germination | Spore Germination with 10% Hardwood Extract | Modified PDA |
|---|---|---|---|
| PDA Difco™ | - | - | 39g |
| Dextrose | 1g | 1g | - |
| Soluble Starch | 1g | 1g | - |
| Yeast Extract | - | - | 2g |

Table 3.1

*Cont.*

| | Spore Germination | Spore Germination with 10% Hardwood Extract | Modified PDA |
|---|---|---|---|
| Agar | 28g | 28g | - |
| Hardwood Extract | 10ml | 100ml | 100ml |
| Water | 990ml | 900ml | 900ml |

### 3.3 Selection of monokaryotic strains for mating

Once the cultures were stabilized, having uniform growth and appearance on PDA, they were subcultured in quadruplicate from the leading edge of mycelial growth to 60mm x 100mm Petri dishes with modified white rot spore germination media containing 10% hardwood extract (Table 3.1). Sterilized (121$^{o}$C for 15 minutes) toothpicks were used to transfer a small amount of inoculum (1-2 mm) to the center of each Petri dish. The growth rate of each monokaryon was determined at the temperature for which its parental strain was initially reported to have a superior mycelial growth rate. Based on analysis of these results using ANOVA followed by Duncan's multiple range test (SAS 9.2), ten single spore isolates were chosen from each parent. The monokaryotic strains chosen were randomly selected from the ranked groupings provided from the results of the Duncan's multiple range tests. For each parent strain four single spore isolates (SSIs) were selected from the group with the fastest growth rates, three SSIs were selected from the group with the median growth rates, and three SSIs were selected from the group with the slowest growth rates. The purpose of selecting SSIs from these three groupings was to ensure that there would be a variety of growth rates for use in the regression analysis between the mycelial growth rates of the gametes and that of their progeny. Additionally,

selecting individuals from these groups provides a greater level of genetic diversity for the phenotype of mycelial growth rate. Selection resulted in a total of sixty monokaryotic cultures. The isolated monokaryotic cultures and their parent strains are shown in Table 4.1.

### 3.4 Mating of single spore monokaryotic cultures

The 60 single spore monokaryotic cultures were crossed in all possible combinations with their sibling monokaryotic cultures (intrastrain crosses) and with monokaryotic cultures from the other parent strains (interstrain crosses) for a total of 1770 crosses. A wedge of inoculum, approximately 5mm x 10mm, from each of two of the monokaryotic cultures being crossed was placed approximately 30mm apart on modified PDA medium (Table 3.1). Plates with the paired monokaryotic cultures were incubated at room temperature and allowed to grow together. Mycelia from the contact zone were observed at 40x using a light microscope and the squash mount technique. The presence of multiple clamp connections was used as verification that a successful mating event occurred. Plates where successful mating occurred were subcultured from the region where the two cultures met and transferred to a new Petri dish with PDA. Lack of clamp connections was used as verification that mating was not successful. If the result was not clear, i.e. one or two clamp connections were observed but they were not abundant, then the culture was allowed to incubate further and reexamined. If the result remained in question after reexamination then the plate was subcultured from the region where the two cultures met, transferred to a new plate with PDA and followed by a suitable incubation period. In some cases somatic incompatibility was clear after the incubation period because the two monokaryons grew away from each other at the point of inoculation. In these cases the result was scored as a negative mating event. Otherwise the culture was reexamined microscopically for clamp connections. If multiple clamp connections were observed the result was scored as a positive

mating event and the plate was subcultured to a new plate with PDA. If clamp connections were not found the result was scored as a negative mating event.

### 3.5 Mating type identification

Charts in the form of a grid were used to track and verify the putatively labeled mating types of the monokaryons. Mating types were labeled starting with one monokaryon involved in a successful intrastrain cross and labeling it as a putative $A_1B_1$ mating type. This monokaryon was then used as a tester strain. Based on the positive mating with this tester strain monokaryons of opposite mating type ($A_2B_2$) could be identified. Strains that were identified as $A_2B_2$ could then be used to identify the remainder of the $A_1B_1$ strains. A second tester strain was selected from those monokaryons not involved in the first two rounds of mating type identification. This tester strain was labeled $A_2B_1$. Following the same technique, the strains of opposite mating type ($A_1B_2$) could be identified. By using one of the newly identified $A_1B_2$ monokaryons the remainder of the $A_1B_2$ strains could then labeled.

### 3.6 Mycelial growth rate

Five hundred and sixty six dikaryotic cultures isolated from the mating study, the 6 parental strains and the 60 monokaryotic cultures used in the mating study were subcultured and maintained on 60mm x 100 mm Petri dishes with PDA. For each of these cultures a sterilized toothpick (121$^{o}$C for 15 minutes) was used to transfer a small amount of inoculum (1-2 mm) to the center of twenty 60mm x 100mm Petri dishes with PDA from the leading edge of mycelial growth. These plates were then incubated at five temperatures (10$^{o}$C, 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C) with four replications at each temperature. Plates were monitored to determine the number of days for the organism to reach both ends of the diameter of a plate. This is a distance of 5.5

cm. The number of days to achieve this distance was recorded and used to calculate the growth rate. The growth rates of the faster growing cultures incubated at 10$^{o}$C were calculated in this manner but, in the case of slow growing organisms where the constraint of excessive time was an issue, mycelial growth rates were calculated by measuring the diameter of mycelial growth in two perpendicular directions divided 2 and then divided by the number of days since inoculation (Mata et al., 2001). Both methods produced data in the form of mycelial growth rates (mm/day).

This work uses a system based on statistically significant ($p > .05$ ) differences in mycelial growth rates at 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C to categorize strains into cool, warm, mid, or wide temperature strains. These are the same four groupings traditionally used but this system is based on incubation temperature and mycelial growth rate as opposed to fruiting temperature optima. This classification system is proposed for three reasons. First, regardless of cultivation system, *L. edodes* will spend the majority of its time in the vegetative growth phase of its life cycle. Classification based on the predominant life cycle stage of the organism makes intuitive sense and will provide useful information to cultivators. Second, classification of strains based on mycelial growth rate temperature profiles provides methodology for future research which can more easily be standardized when compared to control and standardization of the many factors involved in fruit body production. Factors which affect fruit body production are temperature, humidity, light, length of incubation prior to fruiting, $CO_2$ and $O_2$ concentrations. These environmental factors will vary between different labs, researchers and fruiting rooms. A model based on defined media and a set of factors which are easy to control will eliminate much of the environmental variance and allow for better comparison between researchers. Third, classification of strains based on mycelial growth rate is more expedient than classification based

on fruiting temperature optima. The need to produce fruit bodies is eliminated so the time needed for classification is reduced.

The classification method based on mycelial growth rate uses data collected at 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C and makes comparisons between these data to determine classification into 1 of 4 groups, cool, mid, wide or warm. The criteria used to make classifications based on mycelial growth rates are presented below.

Cool temperature:

If the mycelial growth rate is significantly greater at 15$^{o}$C and 20$^{o}$C than it is at 30$^{o}$C then the strain is classified as a cool temperature strain.

Mid temperature:

If the mycelial growth rate is significantly greater at 20$^{o}$C than at 30$^{o}$C and the growth rate at 15$^{o}$C is significantly different from the growth rate at 30$^{o}$C then the strain is a classified as a mid temperature strain.

Wide temperature:

If the mycelial growth rate at 15$^{o}$C and/or 20$^{o}$C is not significantly different than it is at 30$^{o}$C then the strain is classified as a wide temperature strain.

Warm temperature:

If the mycelial growth rate at 30$^{o}$C is significantly greater than it is at 15$^{o}$C and 20$^{o}$C then the strain is classified as a warm temperature strain.

**3.7 Experimental design and statistical analysis**

A critical rejection value of 5% was used to determine significance for all tests. All growth rate experiments had factorial structured treatments with completely randomized designs and four replications; although due to space and time constraints it was not possible to test all strains at the same time. Every effort was made to identify and mitigate potential sources for confounding effects between inoculation dates. Statistical analyses were performed using SAS 9.2 software for chi square, regression analysis, analysis of variance (ANOVA) or general linear model (GLM) procedures and paired comparisons were performed using Duncan's multiple range test (SASInstitute, 1985). The ANOVA procedure was used when testing between groups with equivalent numbers of data points per treatment. The GLM procedure was used when the numbers of data points per treatment were not equivalent, e.g. when comparing the growth rates from aggregated full sibling groups in which there were different numbers of full siblings in the groups. Duncan's multiple range tests were used to determine significant differences between treatment means. Relationships between the dikaryotic growth rates of novel strains and the mean of the growth rates of their monokaryotic constituents was determined by regression analysis. Differences between expected and observed allele frequencies were determined by chi square test.

Comparisons between the growth rates of novel strains were made in three ways:

1. The first comparisons were made between each full sibling group of novel strains for all incubation temperatures ($10^{o}$C, $15^{o}$C, $20^{o}$C, $25^{o}$C and $30^{o}$C). Full sibling groups were comprised of the novel strains produced by crossing monokaryons from the same two parents, in the case of interstrain groups, or monokaryons from the same parent, in the case of intrastrain full sibling groups.

2. The second set of comparisons was made between all novel strains produced by interstrain crosses and all novel strains produced by intrastrain crosses for each incubation temperature ($10^{o}$C, $15^{o}$C, $20^{o}$C, $25^{o}$C and $30^{o}$C).
3. The third set of analyses was made comparing the growth rates of each strain within each full sibling group to the growth rate(s) of their parents at each incubation temperature ($10^{o}$C, $15^{o}$C, $20^{o}$C, $25^{o}$C and $30^{o}$C).

# CHAPTER 4

## Results and Discussion of Monokaryotic Mating Type and the Effect of Monokaryotic Growth Rates on Dikaryotic Growth Rates

### 4.1 Selection of monokaryons from parent strains

From each of the six parent strains 37 monokaryons were isolated from the germinated spores. From each group of 37 single spore isolates (SSIs) ten were selected for use in breeding crosses. Table 4.1 shows the lineage and relative growth rates, at the temperature for which its parental strain was initially reported to have a superior mycelial growth rate, of the selected monokaryotic SSIs.

Table 4.1

*Isolated monokaryotic cultures listed by parent strain and their relative growth rate*

| | | Parent Strains | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| Identification numbers and relative growth rates of monokaryotic cultures | Fastest | 1 | 11 | 21 | 31 | 41 | 51 |
| | | 2 | 12 | 22 | 32 | 42 | 52 |
| | | 3 | 13 | 23 | 33 | 43 | 53 |
| | | 4 | 14 | 24 | 34 | 44 | 54 |
| | Median | 5 | 15 | 25 | 35 | 45 | 55 |
| | | 6 | 16 | 26 | 36 | 46 | 56 |
| | | 7 | 17 | 27 | 37 | 47 | 57 |
| | Slowest | 8 | 18 | 28 | 38 | 48 | 58 |
| | | 9 | 19 | 29 | 39 | 49 | 56 |
| | | 10 | 20 | 30 | 40 | 50 | 60 |

### 4.2 Novel strains produced by interstrain and intrastrain breeding

A total of 566 novel *L. edodes* offspring were produced by intra and inter crossing of the ten monokaryotic cultures from each of the six parental strains. Twenty two strains were the result of intrastrain crosses, mating of monokaryons from the same parent, and 544 of these strains were the result of interstrain crosses, mating of monokaryons from different parents. The numbers of novel strains produced from intra and inter mating of all selected monokaryons aggregated by full sibling groups are depicted in Figures 4.1 and 4.2 respectively.

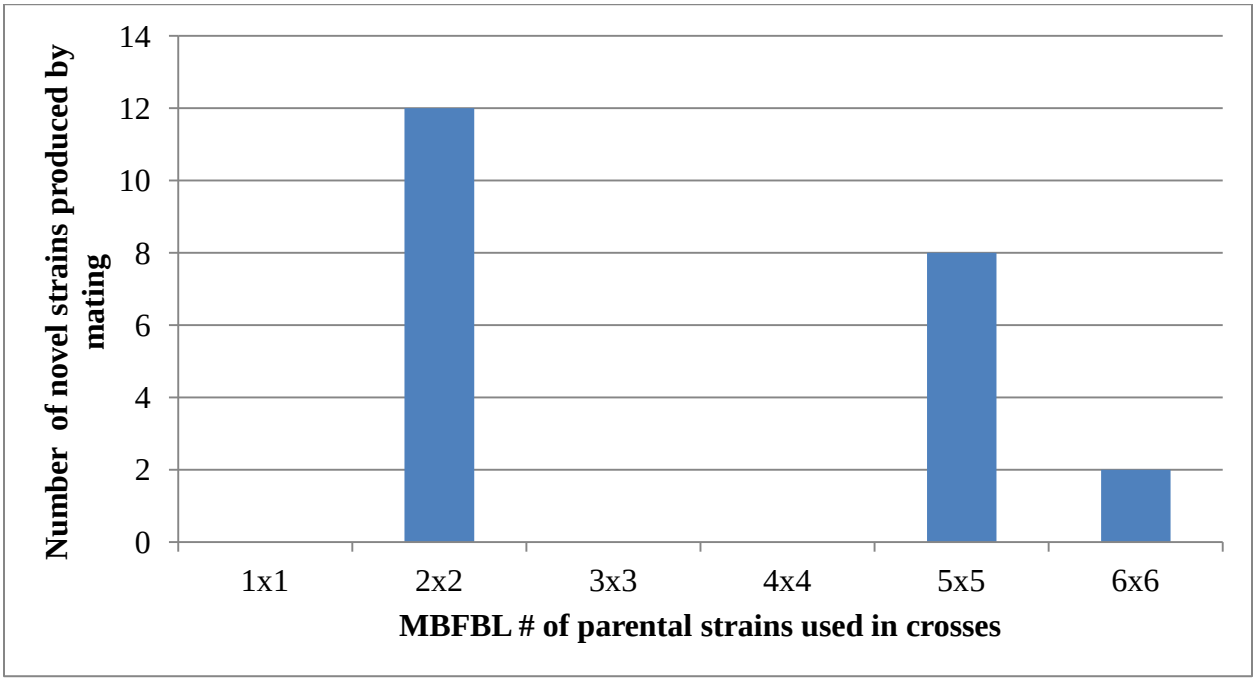



*Figure 4.1.* Novel strains produced by intrabreeding of strains 1, 2, 3, 4, 5 and 6.

Successful mating events were observed in 3 of 6 intrabred groups. Each set of crosses was comprised of a total of 45 combinations of SSIs. Of the strains with successful mating events Mushroom Biology and Fungal Biotechnology Lab (MBFBL) 2, 5 and 6 had a 26.7%, 17.8 %, and 4.4% successful self crossing rates respectively while, MBFBL 1, 3 and 4 had 0.0% successful self crossing rates. The intrastrain crossing rates for 2 and 5 are close to the expected 25% successful self crossing rate while the others are well below the expected rate. The three

strains which did not self cross in this systematic mating program did self cross in mass matings of germinated monokaryotic spores where hundreds to thousands of germinated spores from a given parent were added to single sterile supplemented sawdust substrate block. That successful mating was observed in these supplemented sawdust blocks shows that these strains are in fact self fertile although no successful mating was observed in the controlled pairings. The lower than expected successful mating rate in the strains 1, 3, 4, 5 and 6 suggests that there is a distorted mating type ratio in the population spores from these strains.

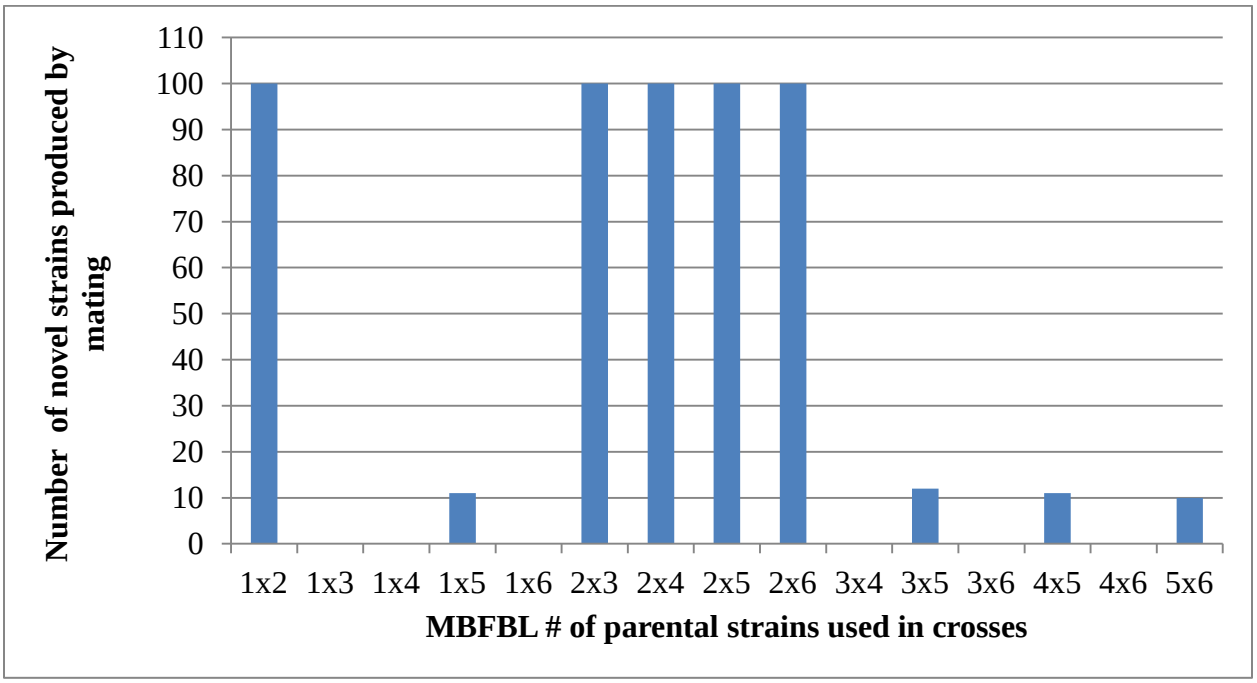



*Figure 4.2.* Novel strains produced by interbreeding of strains 1, 2, 3, 4, 5 and 6.

A total of 1500 interstrain crosses of monokaryons were made. The majority, 400 of 422, of the successful interstrain crosses were produced by crossing monokaryons from MBFBL 2 with monokaryons from the MBFBL 1, 3, 4, 5 and 6 as seen in Figure 4.2. The 100% successful breeding rate between the monokaryons of MBFBL 2 and all other monokaryons is evidence that this strain has A and B mating type alleles that are different from those of the other five strains.

This finding is consistent with the tetrapolar mating system (Kendrick, 2000; E. Kothe, 2001; Raper, 1966).

### 4.3 Identification of mating types

Successful crosses or positive mating events exhibited the presence of abundant clamp connections in hyphae of mated cultures when observed by microscopic examination. The labeling of mating types was tracked and verified with the aid of the construction of mating grids. Analysis of the mating grids from the intrastrain mating of monokaryons was used to identify the mating types present in each of these crosses. The intrastrain mating grid used for the identification of monokaryons from parent strain MBFBL 2 is shown in Figure 4.2.

Intrastrain mating grids were also constructed and used for the initial identification of monokaryons from MBFBL 5 and 6 as these were the only other strains with successful intrastrain mating. Successful mating in a bifactorial tetrapolar system requires that monokaryons be heterozygous at both the A and B alleles.

Table 4.2

*Intrastrain mating grid for monokaryons from MBFBL 2*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 11 $A_4B_3$ | 12 $A_4B_4$ | 13 $A_3B_3$ | 14 $A_¾B_4$ | 15 $A_4B_4$ | 16 $A_4B_4$ | 17 $A_3B_4$ | 18 $A_3B_4$ | 19 $A_3B_3$ | 20 $A_3B_4$ |
| 11 $A_4B_3$ | | - | - | + | - | - | + | + | - | + |
| 12 $A_4B_4$ | - | | + | - | - | - | - | - | + | - |

*Note*. Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol.

Table 4.2

*Cont.*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 11 $A_4B_3$ | 12 $A_4B_4$ | 13 $A_3B_3$ | 14 $A_{¾}B_4$ | 15 $A_4B_4$ | 16 $A_4B_4$ | 17 $A_3B_4$ | 18 $A_3B_4$ | 19 $A_3B_3$ | 20 $A_3B_4$ |
| 13 $A_3B_3$ | - | + | | + | + | + | - | - | - | - |
| 14 $A_{¾}B_4$ | + | - | + | | - | - | - | - | + | - |
| 15 $A_4B_4$ | - | - | + | - | | - | - | - | + | - |
| 16 $A_4B_4$ | - | - | + | - | - | | - | - | + | - |
| 17 $A_3B_4$ | + | - | - | - | - | - | | - | - | - |
| 18 $A_3B_4$ | + | - | - | - | - | - | - | | - | - |
| 19 $A_3B_3$ | - | + | - | + | + | + | - | - | | - |
| 20 $A_3B_4$ | + | - | - | - | - | - | - | - | - | |

*Note*. Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol.

Table 4.3 lists the mating type of monokaryons from MBFBL 1, 3, 4, 5 and 6 including those found to have recombinant mating types. The monokaryons from these five parent strains were found to have the same set of mating alleles, $A_{1or2}$ and $B_{1or2}$. Therefore a single mating grid for all the monokaryons from these strains was constructed showing positive mating events and the mating type of the monokaryons from these parents. The final and complete mating grid used for identification of the mating types of these 50 monokaryons is given in Appendix A, Table A4.1. The ten monokaryons from MBFBL 5 and monokaryon 52 from MBFBL 6 are the only

monokaryons listed in the horizontal axis of this mating grid. This is because these were the only monokaryons which were involved in either interstrain or intrastrain crosses.

Table 4.3

*Mating type of monokaryons with $A_{1or2}$ $B_{1or2}$ mating types*

| | Mating type of monokaryons from MBFBL 1, 3, 4, 5, and 6 | | | | | |
|---|---|---|---|---|---|---|
| | $A_1B_1$ | $A_2B_2$ | $A_1B_2$ | $A_2B_1$ | $A_{½}B_2$ | $A_2B_{½}$ |
| Identification number of monokaryon | 5 | 45 | | 1 | 49 | 52 |
| | 6 | | | 2 | | |
| | 8 | | | 3 | | |
| | 21 | | | 4 | | |
| | 22 | | | 7 | | |
| | 24 | | | 9 | | |
| | 27 | | | 10 | | |
| | 28 | | | 23 | | |
| | 30 | | | 25 | | |
| | 31 | | | 26 | | |
| | 34 | | | 29 | | |
| | 35 | | | 32 | | |
| | 36 | | | 33 | | |
| | 38 | | | 37 | | |
| | 41 | | | 39 | | |
| | 42 | | | 40 | | |
| | 43 | | | 44 | | |
| | 47 | | | 46 | | |
| | 50 | | | 48 | | |
| | 55 | | | 51 | | |
| | 56 | | | 53 | | |
| | 57 | | | 54 | | |
| | 59 | | | 58 | | |
| | 60 | | | | | |

Table 4.4 shows the mating type of monokaryons from MBFBL 2 including monokaryon 14 where a recombinant mating type was observed. The mating type of these monokaryons are presented in a separate table because they were found to have a unique set of mating types compared to the other 50 monokaryons shown in Table 4.3 above.

Table 4.4

*Mating type of monokaryons with $A_{3or4}$ $B_{3or4}$ mating types*

| | Mating type of monokaryons from MFBBL 2 | | | | |
|---|---|---|---|---|---|
| | $A_3B_3$ | $A_4B_4$ | $A_3B_4$ | $A_4B_3$ | $A_{¾}B_4$ |
| Identification number of monokaryon | 13 | 12 | 17 | 11 | 14 |
| | 19 | 15 | 18 | | |
| | | 16 | 20 | | |

In matings of monokaryons with the same mating type alleles three monokaryons were observed mating with monokaryons carrying either of the binary options for mating type. An example of this situation was observed in the A locus of monokaryon 14 from MBFBL 2 as shown in Table 4.2. Monokaryon 14 successfully mated with monokaryons with both the $A_3$ and $A_4$ mating types as long as that monokaryon had a $B_3$ allele at the B locus. The nomenclature of these recombinant mating types is given as a fraction comprised of the two parental mating types. Based on these observations monokaryon 14 was labeled $A_{¾}B_4$. It is theorized that the recombination which occurred in this mating locus is between complete subloci at the A locus. When complete subloci are recombined the resulting monokaryon can have a main locus which is now able to produce transcription factors which will start the signal cascade in either of the parental mating types. This is functionally a new mating type. This model is supported by the findings of L. A. Casselton (1997); Fox et al. (1994); James et al. (2004); Erika Kothe (1996) and Cheng and Lin (2008). Recombinant mating types were observed in monokaryons from MBFBL 5 and 6 as well.

Of the three monokaryons identified with recombinant mating types, monokaryon 14 was found to have complete functionality of both parental A mating alleles while the other two,

monokaryons 49 and 52, exhibited recombinant yet incomplete functionality in the parental A and B mating alleles respectively. Monokaryon 49 from parent MBFBL 5 was found to have a recombinant A mating type but mated only with monokaryons having an $A_2B_1$ mating type for all intrastrain matings. Based on these observations this monokaryon was initially labeled as $A_1B_2$. However when monokaryon 49 was used in interstrain crosses it was found that it successfully crossed with 7 out of 24 of the monokaryons with an $A_1$ mating type and 17 out of 24 of the monokaryons with an $A_2$ mating type. Based on these observations monokaryon 49 was relabeled $A_{½}B_2$ since it was successfully mating, albeit inconsistently, with monokaryons of both the $A_1$ and $A_2$ mating types as long as the B locus was $B_1$. Monokaryon 52 from MBFBL 6 displayed this same type of inconsistency in successful mating but was found to be recombinant at the B locus. Monokaryon 52 mated with none of its sibling monokaryons with an $A_2B_1$ mating type but did successfully mate with 2 out of 5 of its sibling $A_1B_1$ monokaryons. Additionally, in interstrain crossing monokaryon 52 did not successfully mate with monokaryon 45 which has an $A_2B_2$ mating type. There were no monokaryons identified with an $A_1B_2$ mating type but monokaryon 52 did mate successfully with monokaryon 49 with an $A_{½}B_2$ mating type in interstrain mating. Based on these results monokaryon 52 was labeled as $A_2B_{½}$.

Comparing these three recombinant monokaryons, 14, 49 and 52 it is evident that recombinant mating types can add inconsistency to the theoretical tetrapolar mating system. In cases like monokaryon 14 where the recombinant mating type exhibits the complete functionality of both alleles it can increase the number of successful mating events. Monokaryon 14 mated successfully with three other sibling monokaryons. If the A mating locus had not been recombinant monokaryon 14 would have mated with either monokaryon 11 with an $A_4B_3$ mating type or with monokaryons 13 and 19 with an $A_3B_3$ mating type. Monokaryon 14 was able to

mate with both. In this case the recombinant locus seems to have complete $A_3$ and $A_4$ functionality. The recombinant mating loci in monokaryons 49 and 52 do not have the complete functionality of both mating alleles. Monokaryon 49 successfully mated with all intrastrain $A_2B_1$ monokaryons and none of the intrastrain $A_1B_1$ monokaryons but successfully mated with some of both of these mating types in interstrain crosses. Monokaryon 52 was similar in exhibiting different responses to interstrain and intrastrain mating. Monokaryon 52 mated with some of its $A_1B_1$ sibling monokaryons but none of the $A_1B_1$ monokaryons from other parents.

The recombinant mating type of monokaryon 52 limited the number of successful mating events in both intra and inter strain mating. The recombinant mating type of monokaryon 14 increased the number of successful mating events in intrastrain mating. The recombinant mating type of monokaryon 49 behaved as an $A_1B_2$ mating type in intrastrain crosses but mated with $A_1B_1$ and $A_2B_1$ mating types as well as monokaryon 52 with an $A_2B_{½}$ mating type in interstrain crosses. This resulted in a slightly increased number of successful mating events. The observed responses of these three recombinant mating types may be due to where the crossing over occurred in the genome of the loci. The successful mating pattern of monokaryon 14 indicates that an entire sublocus was transferred while in the other two instances the pattern of successful mating indicates that only part of the sublocus was transferred leaving the new recombinant mating type only partially functional to nonfunctional.

The percentage of monokaryons with a recombinant mating type is shown in Table 4.5. The occurrence of recombinant mating type alleles is neither high nor is it uncommon. The observation of mating types which are recombinant at one of the mating type loci is consistent with the findings of Fox et al. (1994) and Cheng and Lin (2008) in their work with *L. edodes* spores.

Table 4.5

*Observed recombinant mating types and their frequency*

| Parent Strains | Number of monokaryotic cultures observed | Number of monokaryon with recombinant mating types | Locus where recombination occurred | Percent of Recombination |
|---|---|---|---|---|
| 2 | 10 | 1 | A | 10 |
| 1 | 10 | 0 | - | 0 |
| 3 | 10 | 0 | - | 0 |
| 4 | 10 | 0 | - | 0 |
| 5 | 10 | 1 | A | 10 |
| 6 | 10 | 1 | B | 10 |
| Total | 60 | 3 | A and B | 5 |

As noted, monokaryon 14 from MBFBL 2 had complete functionality of both mating type alleles while monokaryon 49 exhibited partial functionality and monokaryon 52 exhibited limited functionality of both mating type alleles. These recombinant mating types have interesting implications for breeding. Gametes like monokaryon 14 and to a lesser extent like monokaryon 49 would be useful in breeding backcrosses. Using monokaryons like these should increase the successful backcrossing rate from the expected 25%. Gametes like monokaryon 52 should probably not be used for breeding stock.

**4.4 Frequency and distribution of mating types**

Based on the lower than expected numbers of successful inter and intra strain mating events and with the identification of monokaryotic mating types completed it was evident that the ratio of mating types among the monokaryons from many if not all of the parent strains was not the expected 1:1:1:1. The distribution of the four mating types of monokaryotic cultures from parental strains 1, 3, 4, 5 and 6 were analyzed in order to determine if the frequency of mating

types was skewed. The ratio of the mating types of the monokaryotic cultures could not be analyzed separately for each strain because of the limitations of the chi square test. One of the requirements for the chi square test is that there should be at least five individuals expected in each class. Since there are four classes of mating type and ten individuals from each parent strain it is expected that there will be 2.5 individuals in each class. However by pooling the 50 individuals from all of the parental strains with the same four mating types a chi square analysis may be performed. These results are shown in Table 4.6.

Table 4.6

*Frequency analysis of monokaryons with $A_{1or2}$ $B_{1or2}$ mating types*

| Strains | Mating type ratio of monokaryons $A_1B_1$:$A_2B_2$: $A_1B_2$:$A_2B_1$ | $x^2$ |
|---|---|---|
| 1 | 3:0:0:7 | n/a |
| 3 | 6:0:0:4 | n/a |
| 4 | 5:0:0:5 | n/a |
| 5 | 5:1:0:3 | n/a |
| 6 | 5:0:0:4 | n/a |
| Total | 24:1:0:23 | 21.13** |

*Note.* * = $x^2_{.05\text{-}3}$= 7.82, ** = $x^2_{.01\text{-}3}$= 11.35

The pooled analysis of the four mating types with an $A_{1or2}$ and $B_{1or2}$ mating type shows that the mating type ratio is skewed with a highly significant $x^2$ value of < .01. By looking at the pooled mating type ratio it is evident that the $A_2B_2$ and $A_1B_2$ mating types are deficient in this population. This type of distorted mating type ratio causes the number of positive mating events to be much lower than expected in both intrastrain crosses and in interstrain crosses between strains with the same set of mating types.

As stated above, a minimum of five individuals in each class are needed to perform a chi square test for frequency distribution. For this reason this analysis could not be conducted on the ten monokaryotic cultures produced from spores from MBFBL 2 or the monokaryons from any of the individual strains. However a chi square test could be performed on the mating alleles for each locus and from each strain as there were only two classes for each of the ten individuals. The chi square test results for the frequency distribution of the mating alleles for each locus of the monokaryons produced by each of the parent strain are shown in Table 4.7.

Table 4.7

*Frequency analysis of mating type alleles for the A and B loci*

| Strains | Ratio of four types of monokaryons $A_xB_x$:$A_yB_y$: $A_xB_y$:$A_yB_x$ | Ratio of A alleles $A_x$:$A_y$ | $x^2$ | Ratio of B alleles $B_x$:$B_y$ | $x^2$ |
|---|---|---|---|---|---|
| 1 | 3:0:0:7 | 3:7 ($A_1$:$A_2$) | 1.60 | 10:0 ($B_1$:$B_2$) | 10.00** |
| 2 | 2:3:3:1 | 5:4 ($A_3$:$A_4$) | 0.11 | 3:7 ($B_3$:$B_4$) | 1.60 |
| 3 | 6:0:0:4 | 6:4 ($A_1$:$A_2$) | 0.40 | 10:0 ($B_1$:$B_2$) | 10.00** |
| 4 | 5:0:0:5 | 5:5 ($A_1$:$A_2$) | 0.00 | 10:0 ($B_1$:$B_2$) | 10.00** |
| 5 | 5:1:0:3 | 5:4 ($A_1$:$A_2$) | 0.11 | 8:2 ($B_1$:$B_2$) | 3.60 |
| 6 | 5:0:0:4 | 5:5 ($A_1$:$A_2$) | 0.00 | 9:0 ($B_1$:$B_2$) | 9.00** |
| Total without 2 | 24:1:0:23 | 24:25 ($A_1$:$A_2$) | 0.02 | 47:2 ($B_1$:$B_2$) | 41.33** |
| Total | 26:4:3:26 | 27:31 ($A_x$:$A_y$) | 0.28 | 50:9 ($B_x$:$B_y$) | 28.49** |

*Note.* * = $x^2_{.05\text{-}1}$ = 3.84, ** = $x^2_{.01\text{-}1}$ = 6.64

The results of the chi square test for the distribution of specific mating alleles at each locus show that there is a highly significant imbalance, $x^{2.} < .01$, in the B mating type alleles among the monokaryotic cultures isolated from MBFBL 1, 3, 4 and 6. In these four strains the spores produced are predominantly carriers of the $B_1$ mating type and there is a severe lack of the $B_2$ mating type alleles within this population. Imbalance in the distribution of mating types was observed in spores from commercial strains of *L. edodes* in the work conducted by Fox et al.

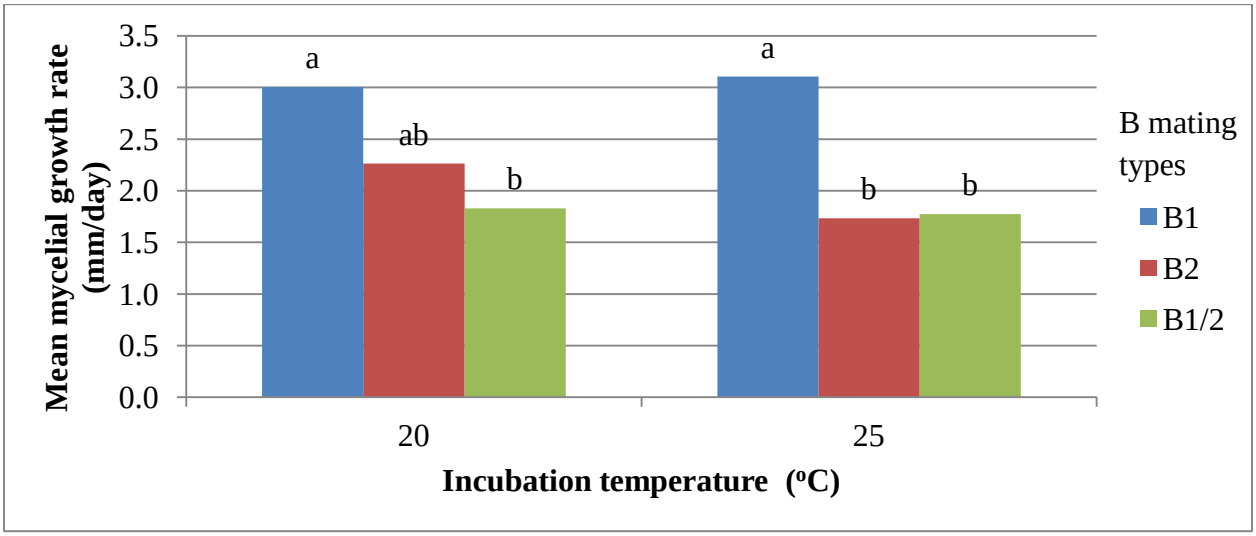
(1994) however the severe lack of a single allele, in this case $B_2$, was not observed. Fox et al. (1994) proposed the existence of a lethal factor as the cause of distorted mating type ratios. This remains a possible explanation for the distorted mating type ratios and the severe lack of the $B_2$ mating type in this population.

**4.5 Comparison of the growth rate of monokaryons based on B mating type alleles**

The growth rates of monokaryons with different B mating types were compared in order to test proposed theories seeking to explain the skewed ratio of B mating type alleles. There are two associated theories for why an imbalanced ratio of mating types may exist in this population of monokaryons. The first is that there is a lethal factor associated with the $B_2$ mating allele which causes spores with this mating type to not germinate and/or grow successfully (Fox et al., 1994). The second is that spores of a given mating type may have a faster growth rate than spores with a different mating type causing a selection bias for monokaryons with the mating type associated with fast growing monokaryons (L. M. Larraya et al., 2001). L. M. Larraya et al. (2001) proposed this theory after observations were made while working with a population of *Pleurotus ostreatus* monokaryons found to have a skewed ratio of mating types. The observance of monokaryons with different mating types growing at different rates has was also reported by Yan et al. (2004) while working with monokaryons isolated from protoplasts of *Stropharia rugoso-annulata*.

The growth rates of monokaryons with different B mating types were compared at 20$^{o}$C and 25$^{o}$C, the two incubation temperatures at which spores were germinated. Results of the statistical analysis of the growth rates of monokaryons with different B mating types are shown below in Figure 4.3. It was found that there was no significant difference ($p < .05$) between the growth rates of monokaryons with $B_1$ and $B_2$ mating types at 20$^{o}$C. There was a significant

difference ($p < .05$) between the growth rates of monokaryons with $B_1$ and $B_2$ mating types at 25°C.




*Note.* Within each temperature, growth rates with the same letter are not significantly different at the 5% level of significance according to Duncan's multiple range tests.

*Figure 4.3*. Growth rates of monokaryons aggregated by common B alleles.

Monokaryons were isolated from plates incubated at both 20°C and 25°C. The analysis of the growth rate of monokaryons with different mating types at different temperatures supports the concept of under selection of monokaryons with a $B_2$ mating type at 25°C but not at 20°C. Secondly, at both 20°C and 25°C there is no significant difference ($p < .05$) between the growth rates of monokaryons with $B_2$ and $B_{½}$ mating types. The theoretical occurrence of the recombinant $B_{½}$ mating type is infrequent compared to the expected occurrence of the $B_2$ mating type which should be present in half of the monokaryons form parents with $B_{1and2}$ mating types, yet this monokaryon was selected. Thirdly, the selection process used for monokaryons involved choosing three of the slowest growing monokaryons from each parent. This should have allowed for selection of monokaryons with $B_2$ mating alleles even if they were slower growing. Finally, plates used for spore germination and isolation of monokaryons were prepared with serial

dilutions of spore slurries. The plates inoculated with the lowest concentrations of spores, where there was little likelihood of chance mating, were left to incubate for up to a month. In these cases, it would seem that slower growing and or germinating monokaryons would have had the opportunity to be selected. These arguments do not support the theory that slow germination and growth led to under selection of monokaryons with a $B_2$ mating type. However there are two issues which must be mentioned that keep the under selection theory from being discarded. The first issue is that since there were only two monokaryons with a $B_2$ mating type, the growth rate analysis comparing monokaryons with different B mating alleles is not as robust as it should be. The second issue is that the plates which were left to incubate for up to one month contained a limited number of germinating spores due to the dilution level of the spore slurries used on these plates. More research to determine the cause of the limited number of monokaryons with a $B_2$ mating type allele and to rule out either of the competing theories of under selection (L. M. Larraya et al., 2001) or lethal factor(s) (Fox et al., 1994; Judelson et al., 1995) must be conducted in order to make a conclusion.

**4.6 Relationship between dikaryotic and parental monokaryotic growth rates**

Mycelial growth rates of dikaryotic cultures were compared to the growth rates of their constituent monokaryons. Results are shown in Table 4.8 below. Regression plots, Figures A4.1 – A4.5, and ANOVA tables, Tables A4.2 – A4.6, for growth rates of monokaryons and dikaryons incubated at $10^oC$, $15^oC$, $20^oC$, $25^oC$ and $30^oC$ are located in Appendix A.

These comparisons were made using the growth rates of organisms incubated at each of the studied incubation temperatures. When the organisms were incubated at $10^oC$ the regression was nonsignificant. When incubated at the other temperatures studied, significant relationships

between the growth rates of parental monokaryons and their dikaryotic progeny were observed. In general, the growth rates of the constituent monokaryons predicted only a small amount, 1-2%, of the variance in the growth rate of the dikaryotic progeny. Models show that the effect of monokaryotic growth rate on the dikaryotic growth rate was also small. These low values indicate that monokaryotic growth rate is not useful as a criterion for selection of monokaryons to be used in strain improvement for mycelial growth rate.

Table 4.8

*Results of linear regression analyses comparing the mean growth rate of constituent monokaryons to the mean growth rate of resulting dikaryons*

| Temperature | Model | $R^2$ | *p* Value |
|---|---|---|---|
| 10°C | - | - | 0.4035 |
| 15 °C | y = 0.128x + 2.290 | 0.01 | 0.0092 |
| 20 °C | y = 0.150x + 3.298 | 0.02 | 0.0008 |
| 25 °C | y = 0.150x + 3.801 | 0.02 | 0.0007 |
| 30 °C | y = 0.256x + 2.249 | 0.01 | 0.0137 |

When incubated at 20°C and 25°C, close to the optimal temperature for *L. edodes*, 2% of the variance in dikaryotic growth rate is explained by the growth rates of the parental monokaryons while at the temperature extremes the coefficient of determination was found to be 1%. The difference in values at optimal and extreme temperatures hint at increasing variation, likely due to an environmental component, which has not been modeled in this study.

The question of what accounts for the majority, 98-99%, of the variance in dikaryotic growth rate remains unanswered. However, data collected may prove useful when coupled with genome sequencing which could lead to the identification of quantitative trait loci as has been done by Luis M Larraya, Alfonso, Pisabarro, and Ramírez (2003) with *Pleurotus ostreatus*.

The null hypothesis, that growth rates of parental constituent monokaryons does not affect the growth rates of the resulting dikaryons, is rejected for growth rates at 15$^{o}$C-30$^{o}$C. However, due to low $R^2$ vales monokaryotic growth rates should not be used as a selection criterion when choosing *L. edodes* monokaryons for breeding dikaryons with improved growth rates. This is in agreement with the findings of Miyazaki (2008) who recommend that monokaryotic growth rates should not be the basis for selection of gametes for breeding although a relationship between the growth rates of *L. edodes* dikaryons and their constituent monokaryons was found. This finding is also in agreement with Simchen and Jinks (1964) who worked with *Schizophyllum commune* and the work of O. S. Isikhuemhen et al. (2010) in studies conducted with *Lentinus squarrosulus*. The heritability of mycelial growth rates from monokaryons to dikaryons may be species dependant as a relationship was reported in *Pleurotus sapidus* by S. S. Wang and Anderson (1972).

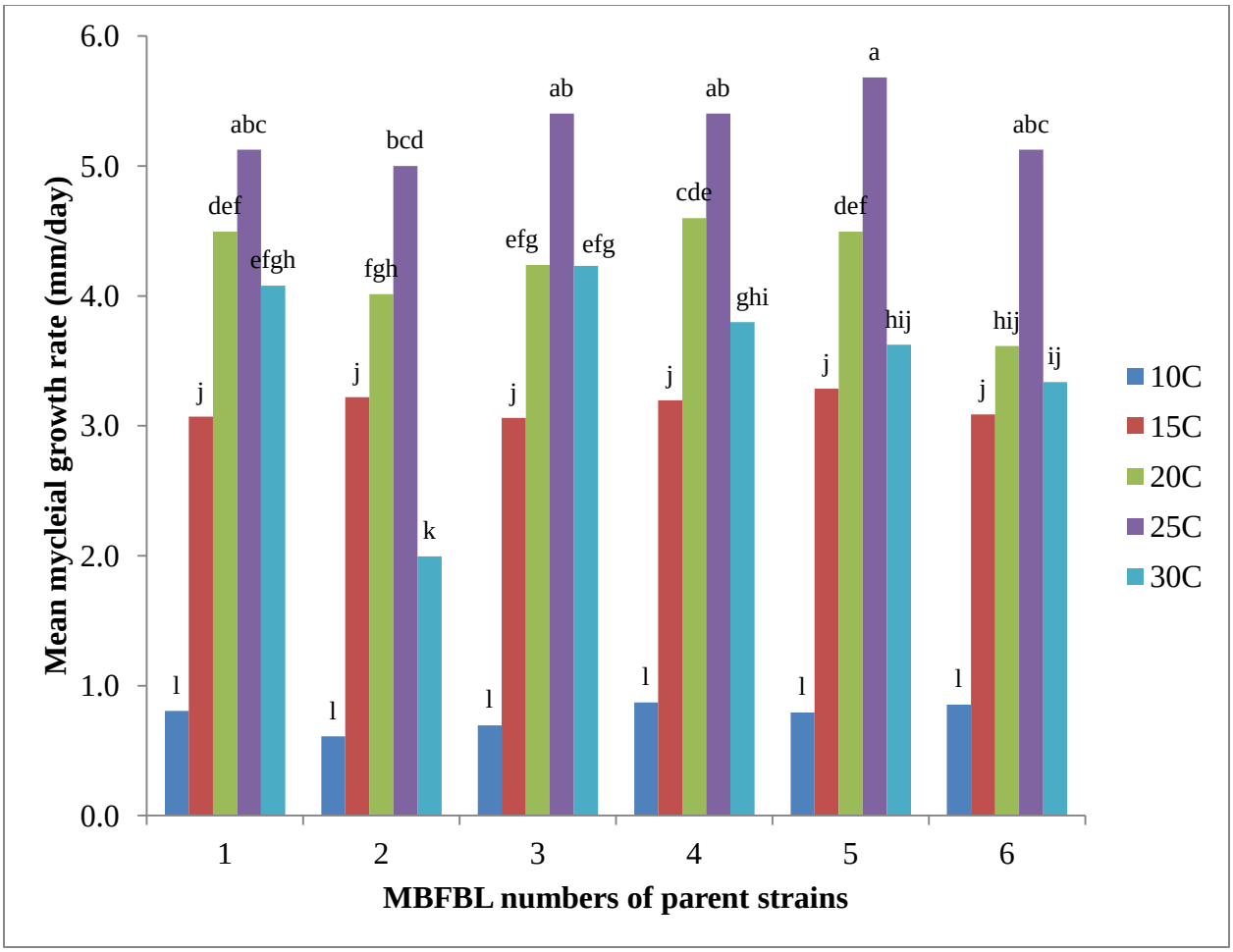
# CHAPTER 5

## Results and Discussion of Mycelial Growth Rates and Temperature Profiles for Parental and Offspring Dikaryons

### 5.1 Mycelial growth rates of parental strains at each incubation temperature

Initial temperature classification of the parental strains was based on previous work completed in the Mushroom Biology and Fungal Biotechnology Lab (MBFBL) which characterized the parent strains as having superior mycelial growth rates at given temperatures when compared to other strains (section 3.1). Initial temperature characterization was also based on antidotal reports of temperatures during occurrences of fruit body formation on inoculated logs. This work reexamined the temperature classification of the six parental strains. The reclassification was based on the growth rates of strains on PDA at 10$^{o}$C, 15$^{o}$C, 20$^{o}$C, 25$^{o}$C and 30$^{o}$C. These data were used to construct mycelial growth rate temperature profiles. Results from ANOVA for the mycelial growth rates at the five incubation temperatures were used to classify each parent as having a cool, mid, warm or wide temperature mycelial growth rate profile.

Results from the study of mycelial growth rates of the six parental strains incubated at five temperatures indicated that there was an interaction effect between strain and temperature. This was as expected as this effect is well understood (Zervakis et al., 2001). ANOVA showed that differences between growth rates were highly significant, $p < .0001$. The ANOVA table is shown in Appendix B, Table B5.1. The average mycelial growth rates of the parent strains at each of the five incubation temperatures are shown in Figure 5.1. The method for characterization of temperature profiles is explained in section 3.6.




*Note.* Growth rates with a letter in common are not significantly different at the 5% level of significance as indicated by Duncan's multiple range tests.

*Figure 5.1.* Mean mycelial growth rates of parent strains incubated at 10°C, 15°C, 20°C, 25°C and 30°C.

The average mycelial growth rate of the parent strains grown at 10°C and 15°C followed similar trends with no significant differences between the strains observed at either temperature. Growth rates at 10°C ranged from 0.61 – 0.87 mm/day. Mycelial growth rates at 15°C ranged from 3.06 – 3.28 mm/day. All strains grew faster at 15°C than at 10°C and growth at 10°C was much slower than all other temperatures. These results indicate that incubation at 10°C is far

enough below the optimum temperature for mycelial growth of *L. edodes* that there is a leveling effect in growth rate among these strains.

All parental strains grew faster when incubated at 25$^{o}$C than any other temperature. This indicated that despite differences in growth rates between strains at other temperatures 25$^{o}$C is the optimum temperature for mycelial growth for these strains. By comparing the mycelial growth rates at temperatures on either side of this optimum, assessments of the temperature profiles of the parent strains were made and the parent strains were classified as a warm, cool, mid or wide temperature strains. The mycelial growth rate temperature characterizations of the parental strains using this classification system are shown in Table 5.1.

Table 5.1

*Temperature classifications of parent strains based on mycelial growth rates at 15-30$^{o}$C*

| MBFBL # | Temperature classification | Rational for classification |
|---|---|---|
| 1 | wide temperature | $20^{o} = 30^{o}$C |
| 2 | cool temperature | $15^{o}$ and $20^{o} > 30^{o}$C |
| 3 | wide temperature | $20^{o} = 30^{o}$C |
| 4 | mid temperature | $20^{o} > 30^{o}$C |
| 5 | wide temperature | $15^{o} = 30^{o}$C |
| 6 | wide temperature | $15^{o} = 20^{o} = 30^{o}$C |

*Note.* = indicates no significant difference between growth rates and > indicates a significant difference with a higher mean indicated by the direction of the sign, at the 5% level of significance as indicated by Duncan's multiple range tests.

The results of the temperature classifications of the parental strains differ in some respects from their initial temperature classifications based on previous work conducted in the MBFBL. MBFBL 1 was initially classified as a cool temperature, 10$^{o}$C strain. The mycelial growth rate of MBFBL 1 was found to be no different at 10$^{o}$C or 15$^{o}$C than any of the other strains and MBFBL 1 was among the strains with the fastest growth at 30$^{o}$C. MBFBL 2 while not faster at its indicated temperature of 10$^{o}$C or the next coolest temperature studied, 15$^{o}$C, than

any of the other parent strains was found to be a cool temperature strain as originally indicated. MBFBL 3 was not found to be superior at 15$^{o}$C as initially indicated and was classified as a wide temperature strain. MBFBL 4 was among strains with the highest growth rates at 20$^{o}$C as determined in previous work. Similarly, MBFBL 5 was among strains with the highest growth rates at 25$^{o}$C as determined in previous work. MBFBL 6 was not among the strains with the highest growth rates at 30$^{o}$C as had been previously determined. The temperature profiling method used in this work provides broader information about the response to temperature of these strains than was previously available.

**5.2 Mycelial growth rates and temperature profiles aggregated by full sibling groups**

The technique used to classify parental strains with the mycelial growth rate temperature profiles was used on aggregated full sibling groups of novel strains. The mean mycelial growth rates used for constructing mycelial growth rate temperature profiles for the 12 full sibling groups are shown in Figure 5.2. Data used for constructing temperature profiles was analyzed by GLM followed by Duncan's multiple range tests. The ANOVA table is shown in Appendix B, Table B5.2.

The majority of the 12 full sibling groups were found to have cool temperature mycelial growth rate temperature profiles. This was true even for those full sibling groups without a parent which was classified as a cool temperature strain i.e. those full sibling groups that do not have MBFBL 2 as a parent. The converse is also true. Full sibling groups, 1x2, 2x3, and 2x6 all have mycelial growth rate temperature classifications of wide although they involve gametes from the cool temperature characterized MBFBL 2. Table 5.2 shows the basis for determination

of the mycelial growth rate temperature classification and the classification of the aggregated full sibling groups.

Table 5.2

*Temperature classifications of full sibling mating groups based on mycelial growth rates at 15-30$^{o}$C*

| Full sibling groups identified by MBFBL # of parents | Mean temperature classification of full sibling group | Rational for classification |
|---|---|---|
| 1x2 | wide temperature | $25^{o}C > 20^{o}C > 15^{o}C = 30^{o}C$ |
| 1x5 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 2x3 | wide temperature | $25^{o}C > 20^{o}C > 15^{o}C = 30^{o}C$ |
| 2x4 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 2x5 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 2x6 | wide temperature | $25^{o}C > 20^{o}C > 15^{o}C = 30^{o}C$ |
| 3x5 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 4x5 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 5x6 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 2x2 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 5x5 | cool temperature | $25^{o}C > 20^{o}C > 15^{o}C > 30^{o}C$ |
| 6x6 | warm temperature | $25^{o}C = 30^{o}C > 20^{o}C$ |

*Note*. = indicates no significant difference between growth rates and > indicates a significant difference with a higher mean indicated by the direction of the sign, at the 5% level of significance as indicated by Duncan's multiple range tests.

Graphic representation of the above information as well as the AVOVA table can be seen in Figures 5.2 below and Table B5.2 in Appendix B respectively. It has been asserted, using classifications based on fruit body development, warm and mid temperature strains have a dominant phenotype (Hasebe et al., 1998; Ryu et al., 2009). This was not observed in temperature classifications based on mycelial growth rates. Table 5.3 shows that dominance of high temperature classifications of warm or wide temperature, were observed in 4 of 12 full sibling groups while cool temperature dominance was observed in 8 of 12 full sibling groups.

Table 5.3

*Temperature classifications of parent strains compared to average classifications of full sibling mating groups*

| MBFBL # of parents | Temperature classification of parents | Mean temperature classification of sibling mating group |
|---|---|---|
| 1x2 | wide x cool | wide temperature |
| 1x5 | wide x wide | cool temperature |
| 2x3 | cool x wide | wide temperature |
| 2x4 | cool x mid | cool temperature |
| 2x5 | cool x wide | cool temperature |
| 2x6 | cool x wide | wide temperature |
| 3x5 | wide x wide | cool temperature |
| 4x5 | mid x wide | cool temperature |
| 5x6 | wide x wide | cool temperature |
| 2x2 | cool x cool | cool temperature |
| 5x5 | wide x wide | cool temperature |
| 6x6 | wide x wide | warm temperature |

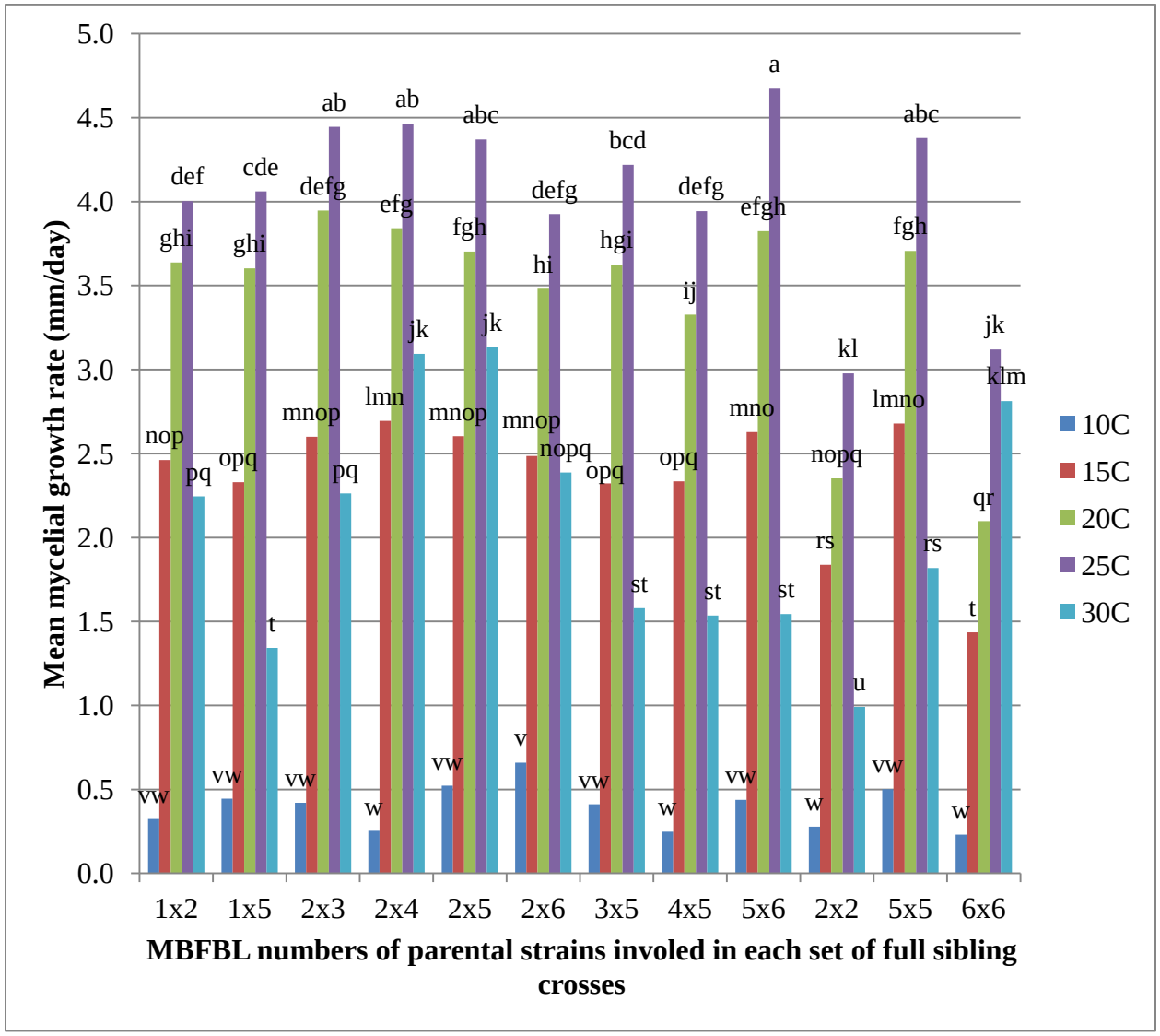
In order for a direct comparison of these results to the results of Hasebe et al. (1998) and Ryu et al. (2009), who have identified dominant phenotypes based on fruiting temperature, a comparison must be made and a relationship found between fruiting temperature based temperature profiles and mycelial growth rate based temperature profiles. This comparison would add support to the work of B. Wang et al. (2004) who has noted such a relationship. However, the results of this work indicate that there is not a clear hierarchy of dominance for mycelial growth rate temperature classifications as has been found for optimum fruiting temperature based classifications.

**5.3 Mycelial growth rates of interstrain and intrastrain crosses**

Mycelial growth rate is a quantifiable phenotype and a measure of vigor which may be used to test the hypothesis that outcrossing or hybridization between strains of the same species will increase vigor compared to crossing monokaryons from the same strain. This phenomenon is known as heterosis. Figure 5.2 shows the average growth rates of the nine interstrain full sibling mating groups and the three intrastrain full sibling mating groups at 10°C, 15°C, 20°C, 25°C and 30°C. Heterosis has been observed in the breeding of *Pleurotus* sp. by Kinugawa, Tanesaka, Nagata, and Watanabe (1997) and in *Stropharia rugoso-annulata* by Yan and Jiang (2005). It should be noted that the numbers of offspring in each full sibling mating group are different and are shown in Figures 4.1 and 4.2**.**

No statistically significant differences in growth rates were observed between full sibling mating groups when they were incubated at 10°C. When incubated at 15°C, 20°C and 25°C all interstrain full sibling groups had statistically higher growth rates than the intrastrain full sibling mating groups 2x2 and 6x6. The mycelial growth rate of the intrastrain full sibling group 5x5 was not statistically different from the interstrain mating groups at 15°C. There were no significant differences between the growth rates of intrastrain full sibling mating group 5x5 and all other interstrain full sibling mating groups at 20°C except for full sibling group 4x5 which was found to be significantly slower than the 5x5 full sibling mating group. Intrastrain full sibling mating group 5x5 had a significantly greater mycelial growth rate than full sibling groups 4x5, 2x6, and 1x2 when incubated at 25°C. When incubated at 30°C, the intrastrain full sibling group 6x6 was among the full sibling groups with the fastest mycelial growth rates along with interstrain full sibling groups 2x5 and 2x4. Interstrain full sibling groups 1x2, 2x3 and 2x6 were found to have the next fastest growth rates at 30°C, followed by intrastrain full sibling mating group 5x5 and interstrain full sibling groups 3x5, 4x5, and 5x6. These interstrain full sibling

groups did not have significantly different growth rates from interstrain full sibling group 1x5. Intrastrain full sibling group 2x2 was found to have the slowest growth rate at 30°C.




*Note.* Growth rates with a letter in common are not significantly different at the 5% level of significance as indicated by Duncan's multiple range tests.

*Figure 5.2.* Mean mycelial growth rates of each full sibling group of novel offspring produced by interbreeding and intrabreeding monokaryons of different parental strains.

Interstrain full sibling mating groups tended to have higher growth rates than 2 of the 3 intrastrain full sibling mating groups at the studied incubation temperatures; however there are

the exceptions noted above as well as the lack of significant differences at the 10°C incubation temperature and no such trend at 30°C. Based on the performance of full sibling group 5x5 and full sibling group 6x6 at 30°C it must be concluded that the case for increased vigor as measured by mycelial growth rate of interbred crosses when compared to inbred crosses is not entirely clear. There are some instances where strains produced by intrabreeding have, in aggregated full sibling groups, growth rates greater than or equal to strains produced by interbreeding. The ANOVA table for these results is shown in Appendix B, Table B5.2.

To further examine the hypothesis that outcrossing will produce strains with higher mycelial growth rates than those produced by inbreeding, the growth rates of strains produced by each method were compared for differences at each temperature. These results are shown in Table 5.4 below.

Table 5.4

*Comparison of mycelial growth rates of novel strains produced by inter and intra strain crosses*

| Incubation Temperature | Mean mycelial growth rate of novel strains from interstrain crosses | Mean mycelial growth rate of novel strains from intrastrain crosses | *p* value |
|---|---|---|---|
| 10°C | 0.47 | 0.40 | 0.21 |
| 15°C | 2.78 | 2.27 | <.0001 |
| 20°C | 3.99 | 3.30 | <.0001 |
| 25°C | 4.53 | 3.76 | <.0001 |
| 30°C | 2.77 | 1.60 | <.0001 |

*Note. ANOVA was performed for each temperature.*

The mycelial growth rates of *L. edodes* strains produced by interbreeding monokaryons from different strains was significantly higher than the mycelial growth rates of strains produced by intrabreeding monokaryons from the same strain at all incubation temperatures except 10°C.

There was no significant difference between mycelial growth rates of strains produced by interbreeding and intrabreeding at 10$^{o}$C. The next level of analysis examines what is perhaps a more important question; does either interbreeding or intrabreeding produce strains which have higher mycelial growth rates than their parents?

### 5.4 Identification of novel strains with mycelial growth rates superior to their parents

In order to assess the success of breeding for increased mycelial growth rates of *L. edodes* strains, the growth rates of novel progeny strains were compared to the growth rates of the their parents. This comparison was made using ANOVA for each incubation temperature and for each of the twelve full sibling mating groups. This allowed for the identification of individual progeny strains which were faster than either one parent or both parents. These results are shown in Tables 5.5 – 5.9.

Table 5.5

*Comparison of parent and offspring mycelial growth rates at 10$^{o}$C*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL #s of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 0.81 | 0.61 | 0.0 | n/a | 1.0 | 1210 |
| 1x5 | 0.81 | 0.79 | 7.7 | 1305 | 7.7 | 1305 |
| 2x3 | 0.61 | 0.70 | 5.0 | 1362, 1368, 1331, 1389, 1364 | 1.0 | 1362 |
| 2x4 | 0.61 | 0.87 | 2.0 | 1443,1444 | 0.0 | n/a |
| 2x5 | 0.61 | 0.79 | 8.0 | 1580, 1583, 1546, 1551, 1582, 1543, 1517, 1573 | 2.0 | 1580,1583 |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Table 5.5

*Cont.*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL #s of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 2x6 | 0.61 | 0.85 | 26.0 | 1623, 1650, 1626, 1673, 1644, 1703, 1705, 1655, 1654, 1663, 1645, 1614, 1651, 1704, 1656, 1672, 1625,1613, 1620, 1696, 1643, 1653, 1664, 1685, 1630, 1670 | 0.0 | n/a |
| 3x5 | 0.70 | 0.79 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 0.87 | 0.79 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 0.79 | 0.85 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 0.61 | 0.61 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 0.79 | 0.79 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 0.85 | 0.85 | 0.0 | n/a | 0.0 | n/a |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Since progeny full sibling groups are comprised of different numbers of individuals results include the percentage of strains which were faster than one or both parent strains. The average growth rate of each parent strain is given in the order listed in the mating group column. Four strains were found to have significantly higher growth rates than both parents when grown at 10$^{o}$C. These strains were produced from crosses between monokaryons from MBFBL 2, a cool temperature strain, and MBFBL 5 and 3, both wide temperature strains and from the cross of MBFBL 1 and 5 both wide temperature strains. The highest percentage of progeny with growth rates superior to both parents at 10$^{o}$C was 7.7%; this percentage was achieved by full sibling

group 1x5. The full sibling group with the next highest percentage of progeny with superior growth rates at 10°C was full sibling group 2x5 with a rate of 2% of progeny with growth rates superior to both parents.

Table 5.6

*Comparison of parent and offspring mycelial growth rates at 15°C*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL #s of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 3.07 | 3.22 | 2.0 | 1222, 1220 | 2.0 | 1222, 1220 |
| 1x5 | 3.07 | 3.29 | 0.0 | n/a | 0.0 | n/a |
| 2x3 | 3.22 | 3.06 | 0.0 | n/a | 2.0 | 1289, 1226 |
| 2x4 | 3.22 | 3.20 | 0.0 | n/a | 0.0 | n/a |
| 2x5 | 3.22 | 3.29 | 3.0 | 1522, 1583, 1566 | 2.0 | 1522, 1583 |
| 2x6 | 3.22 | 3.09 | 3.0 | 1681, 1644, 1684 | 3.0 | 1681, 1644, 1684 |
| 3x5 | 3.06 | 3.29 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 3.20 | 3.29 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 3.29 | 3.09 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 3.22 | 3.22 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 3.29 | 3.29 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 3.09 | 3.09 | 0.0 | n/a | 0.0 | n/a |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Seven strains were found to have significantly greater mycelial growth rates compared to their parents when grown at 15°C. The strains exhibiting superior growth rates were from crosses between MBFBL 2, a cool temperature strain and MBFBL 1, 5, and 6, all wide temperature strains. Only one of these strains, MBFBL 1583, also showed a superior growth rate at 10°C. Three percent of the progeny from full sibling group 2x6 were superior to both parents. This was the highest percentage of progeny with superior growth rates at 15°C.

Table 5.7

*Comparison of parent and offspring mycelial growth rates at 20°C*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 4.50 | 4.01 | 0.0 | n/a | 0.0 | n/a |
| 1x5 | 4.50 | 4.50 | 0.0 | n/a | 0.0 | n/a |
| 2x3 | 4.01 | 4.24 | 1.0 | 1332 | 0.0 | n/a |
| 2x4 | 4.01 | 4.60 | 1.0 | 1424 | 0.0 | n/a |
| 2x5 | 4.01 | 4.50 | 1.0 | 1523 | 0.0 | n/a |
| 2x6 | 4.01 | 3.61 | 1.0 | 1684 | 3.0 | 1684, 1794, 1624 |
| 3x5 | 4.01 | 4.50 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 4.60 | 4.50 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 4.50 | 3.61 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 4.01 | 4.01 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 4.50 | 4.50 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 3.61 | 3.61 | 0.0 | n/a | 0.0 | n/a |

*Note. Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.*

Only strain MBFBL 1684, progeny of MBFBL 2 and 6, cool and wide temperature strains respectively, grew at a higher mycelial growth rate than both parents when grown at 20°C. This strain represents 1% of the progeny of the full sibling group 2x6. MBFBL 1684 showed a superior growth rate at 15°C as well as 20°C, indicating that this strain may be suitable for use at cooler temperatures in the functional temperature range of *L. edodes* and therefore useful in extending the outdoor production season into cooler seasons and reducing the need to heat incubation areas in cooler seasons for indoor production.

Two strains were found to have greater mycelial growth rates than both of their parents when grown at 25°C. Both are progeny of MBFBL 2, a cool temperature strain, and either MBFBL 1 or 6, both wide temperature strains. One of these strains is from full sibling group

Table 5.8

*Comparison of parent and offspring mycelial growth rates at 25°C*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 5.13 | 5.00 | 1.0 | 1220 | 1.0 | 1220 |
| 1x5 | 5.13 | 5.68 | 0.0 | n/a | 0.0 | n/a |
| 2x3 | 5.00 | 5.40 | 1.0 | 1372 | 0.0 | n/a |
| 2x4 | 5.00 | 5.40 | 3.0 | 1424, 1443, 1432 | 0.0 | n/a |
| 2x5 | 5.00 | 5.68 | 1.0 | 1522 | 0.0 | n/a |
| 2x6 | 5.00 | 5.13 | 1.0 | 1684 | 1.0 | 1684 |
| 3x5 | 5.40 | 5.68 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 5.40 | 5.68 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 5.68 | 5.13 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 5.00 | 5.00 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 5.68 | 5.68 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 5.13 | 5.13 | 0.0 | n/a | 0.0 | n/a |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

1x2, representing 1% of the progeny from this full sibling group. The other is from full sibling group 2x6, also representing 1% of the progeny of this full sibling group. As mentioned, MBFBL 1684 demonstrated superior mycelial growth at 15°C, 20°C and 25°C making it a good candidate for further use in production and breeding research.

Fifty four strains were found to have mycelial growth rates greater than both of their parents when incubated at 30°C. Table B5.3 in Appendix B shows the MBFBL numbers of these 54 strains. Strains which were found to have growth rates that were greater than both of their parents at 30°C were from crosses between MBFBL 2, a cool weather strain, and MBFBL 4, 5 and 6, mid, wide and wide temperature strains respectively. It should be noted that MBFBL 1684

Table 5.9

*Comparison of parent and offspring mycelial growth rates at 30°C*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 4.08 | 2.00 | 0.0 | n/a | 32.0 | 32 strains See Appendix B Table B5.3 |
| 1x5 | 4.08 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 2x3 | 2.00 | 4.23 | 31.0 | 31 strains See Appendix B Table B5.3 | 0.0 | n/a |
| 2x4 | 2.00 | 3.80 | 63.0 | 63 strain See Appendix B Table B5.3 | 10.0 | 10 strains See Appendix B Table B5.3 |
| 2x5 | 2.00 | 3.62 | 61.0 | 61 strains See Appendix B Table B5.3 | 26.0 | 26 strains See Appendix B Table B5.3 |
| 2x6 | 2.00 | 3.34 | 46.0 | 46 strains See Appendix B Table B5.3 | 18.0 | 18 strains See Appendix B Table B5.3 |
| 3x5 | 4.23 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 3.80 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 3.62 | 3.34 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 2.00 | 2.00 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 3.62 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 3.34 | 3.34 | 0.0 | n/a | 0.0 | n/a |

*Note. Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests. MBFBL numbers of strains which were found to be significantly faster than either and/or both parents are listed in Table B5.3.*

which was found to have growth rates superior to its parents at 15°C, 20°C and 25°C was not found to have a superior growth rate at 30°C. Several strains which were identified as having superior mycelial growth rates at 30°C were also identified as having superior growth rates at other temperatures as well. Strains 1580 and 1583 products of monokaryons from MBFBL 2 and 5 were found to have growth rates superior to both of their parents at 10°C as well as 30°C. For

strain 1583, this was true at 15°C and 30°C as well as 10°C. Strain 1681, a product of monokaryons from MBFBL 2 and 6 was found to have a growth rate superior to its parent strains at 15°C and 30°C. These strains exhibit growth rates superior to their parents at temperatures both above and below the optimum temperature for mycelial growth of *L. edodes* making them good candidates for further use in production and as breeding stock.

More novel strains with growth rates which were greater than both of their parents were found at 30°C than at any of the other temperatures studied. These strains represented 10% of the progeny from full sibling group 2x4, 26% of the progeny from full sibling group 2x5 and 18% of the progeny from full sibling group 2x6. These are much higher percentages of progeny with superior growth rates than were found at any of the other incubation temperatures. This suggests that breeding for *L. edodes* which can grow well at temperatures higher than the species optimum of 20-28°C, cited by Zervakis et al. (2001), can be achieved. This evidence supports the concept that *L. edodes* strains which perform at higher temperatures can be produced through breeding. Based on the observed results comparing temperature classifications of parents to average temperature classifications of offspring full sibling groups (Table 5.3) the phenotype of superior high temperature tolerance as measured by growth rate, is not dominant in the Mendelian sense. However, the finding that a much larger number of novel strains with growth rates superior to both parents were identified at 30°C suggests that it is an easier phenotype to achieve through breeding than superior growth rate at other temperatures.

All of the strains identified as having mycelial growth rates higher than both of their parents were products of interstrain crosses. This was true for all temperatures studied. This finding strongly supports the hypothesis that interstrain crossing is a better method of increasing vigor than intrastrain crossing. Furthermore, this finding does not support the theory that

backcrossing is a sound method of achieving strains with a superior growth rate at a given temperature.

All of the strains with a growth rate superior to their parents except strain 1210, a product of monokaryons from MBFBL 1 and 5, were from crosses involving monokaryons from MBFBL 2. MBFBL 2 was found to have mating type alleles which were unique at both mating loci compared to the other five parent strains. The presence of unique mating type alleles is an indicator of genetic diversity (Fox et al., 1994; E. Kothe, 2001) and suggests that of the six parent stains MBFBL 2 is the strain which is the least related to the other five strains. That in almost all cases it was monokaryons from MBFBL 2 combined with monokaryons from the other five strains which produced strains with superior growth rates supports the hypothesis that increased genetic diversity in breeding stock will yield novel strains with increased vigor as proposed by Hibbett and Donoghue (1996). This concept of heterosis is also seen in the work of (Kinugawa et al., 1997) with *Pleurotus* sp. and Yan and Jiang (2005) with *Stropharia rugoso-annulata*.

The phenotype of mycelial growth rate seems to be a complex interaction of factors and is not as straight forward as crossing gametes from the fastest growing strains at a given temperature to yield the fastest offspring at that temperature.

# CHAPTER 6

## Conclusions and Future Research

Five hundred and sixty six novel strains were produced through breeding of 60 monokaryons from six strains of *L. edodes*. Five hundred and forty four of these strains were products of interstrain breeding and 22 were the products of intrastrain breeding. This increases the number of strains of *L. edodes* in the MBFBL culture collection. In addition, these strains and the characterization of their growth rates at the five temperatures studied are the first step in a breeding program which will produce strains with superior phenotypes. These strains have a known lineage which makes them useful for future studies using quantitative genetics and molecular techniques for the identification of quantitative trait loci for *L. edodes* phenotypes. The work accomplished in this thesis provides a broad base for future research into the genetics and breeding of the shiitake mushroom.

Strains with growth rates superior to their parents were identified at each temperature studied. Several of these strains had growth rates which were superior to their parents at multiple temperatures. The strategy of continuously screening and selecting strains with mycelial growth rates superior to those of their parents at given temperatures should be continued. All novel strains identified as having growth rates superior to both parent strains were from interstrain crosses. This is strong evidence for the use of interstrain breeding as a strategy for producing strains with superior growth rates.

The comparison of the mean growth rates of aggregated full sibling groups shows it is not the mating of any two monokaryons from a parent or parents that will produce strains with superior growth rates; rather it is the mating of specific monokaryons which will produce strains

with superior growth rates. The results indicate that growth rate is a complex trait and it is the specific combining ability of gametes which produces dikaryons with superior growth rates. This is supported by the finding that mean growth rates of some intrastrain full sibling groups at some temperatures were not statistically significant from the mean growth rates of some interstrain full sibling groups, yet no strains with growth rates superior to their parent were produced by intrastrain crosses. This concept is further supported by the finding that not all interstrain crosses produced offspring with superior growth rates, but all offspring with superior growth rates were the product of interstrain crosses.

A comparison of the mycelial growth rates of monokaryons to their constituent dikaryons in *L. edodes* strains was studied and it was found that monokaryotic mycelial growth rate should not be used as a selection criterion for monokaryons in *L. edodes* when breeding for mycelial growth rate. The determination of monokaryotic growth rate is an unnecessary step and should not be used in future breeding of *L. edodes*.

Though the addition of 566 new strains represents a large increase in the total number of *L. edodes* strains in the MBFBL culture collection and thereby an increase in the number of strains for use by researchers, cultivators and industry, the number of strains produced in this work could have been greater. Two factors played a role in limiting the number of strains produced. The first factor was the finding of common mating type alleles in 5 of 6 parent strains. This limited the majority of the novel strains to offspring of MBFBL 2. The second factor which limited the number of strains produced was the skewed frequency distribution of mating type alleles and a lack of monokaryons with a $B_2$ mating type.

That five of the six parent strains studied had the same set of $A_{1or2}$ and $B_{1or2}$ mating types is an indication that these strains are more closely related to each other than they are to MBFBL 2. Other strains of *L. edodes* in the MBFBL culture collection should be examined to determine if they have the same or different mating types as MBFBL 2 or the other 5 parental strains. This would provide valuable information about the diversity of *L. edodes* in the MBFBL culture collection. If strains with unique mating type alleles or the set of mating type alleles identified in MBFBL 2 are present then they should be incorporated in future breeding efforts.

The finding that the $B_2$ mating type allele was limited in the population of monokaryons from four parent strains and that a fifth strain trended toward the same finding is worthy of further investigation. Two theories, under selection due to slow growth and a lethal factor due to a deleterious mutation, have been proposed. In either case, the existence of the skewed mating type ratio and the limited number of monokaryons with a $B_2$ allele suggests that either these strains are derived from the same lineage where this mutation originated or that the development of a lethal factor involving the $B_2$ allele is a common convergent mutation. If the mating type ratio remains skewed in subsequent generations produced from the offspring of these parent strains then this is evidence that the strains exhibiting a lower than expected frequency of $B_2$ alleles have inherited this factor from a common ancestor and that these strains are closely related. If the frequency of the $B_2$ allele returns to the expected 1:1:1:1 ratio in subsequent generations then this is an indication that the lethal factor is a common occurrence in *L. edodes* which should be addressed prior to or during controlled breeding projects.

Future work is necessary testing the proposed use of temperature designations for *L. edodes* based on mycelial growth rates rather than fruiting temperature optima. Research testing the relationship between these two approaches would be useful as only limited literature is

currently available on the subject. The fruiting temperature based approach to classification is traditional and does provide useful information for the production of mushrooms. However, *L. edodes* will spend the majority of its life growing vegetatively in any production system and there is a role for the mycelial growth rate temperature profile based approach to strain classification. The mycelial growth rate temperature profile based approach provides information allowing the selection of strains suitable for use at temperatures close to ambient climatic conditions, can be used to select strains with high competitive saprobic advantage and can decrease the crop cycle conferring savings of energy and capital to the producer.

While there is much work to continue, the future breeding of *L. edodes* for high growth rates at and across selected temperatures, as well as the continued assessment of genetic diversity of breeding stock, will help to insure that the efficiency of cultivation of this important mushroom will continue to improve in years to come.

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*Appendix A*

Table A4.1

*Mating grid for monokaryons from strains 1, 3, 4, 5 and 6*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 41 $A_1B_1$ | 42 $A_1B_1$ | 43 $A_1B_1$ | 44 $A_2B_1$ | 45 $A_2B_2$ | 46 $A_2B_1$ | 47 $A_1B_1$ | 48 $A_2B_1$ | 49 $A_{½}B_2$ | 50 $A_1B_1$ | 52 $A_2B_{½}$ |
| 1 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 2 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 3 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 4 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 5 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 6 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 7 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 8 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 9 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 10 $A_2B_1$ | - | - | - | - | - | - | - | - | - | - | - |
| 21 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 22 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |

*Note.* Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol. Only monokaryons from MBFBL 5 and monokaryon 52 from MBFBL 6 are shown in the horizontal axis of this table as these were the only monokaryons involved in successful interstrain or intrastrain crosses except for the monokaryons from MBFBL 2.

Table A4.1

*Cont.*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 41 $A_1B_1$ | 42 $A_1B_1$ | 43 $A_1B_1$ | 44 $A_2B_1$ | 45 $A_2B_2$ | 46 $A_2B_1$ | 47 $A_1B_1$ | 48 $A_2B_1$ | 49 $A_{½}B_2$ | 50 $A_1B_1$ | 52 $A_2B_{½}$ |
| 23 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 24 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 25 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 26 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 27 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 28 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 29 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 30 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 31 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 32 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 33 $A_2B_1$ | - | - | - | - | - | - | - | - | - | - | - |
| 34 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 35 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |

*Note.* Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol. Only monokaryons from MBFBL 5 and monokaryon 52 from MBFBL 6 are shown in the horizontal axis of this table as these were the only monokaryons involved in successful interstrain or intrastrain crosses except for the monokaryons from MBFBL 2.

Table A4.1

*Cont.*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 41 $A_1B_1$ | 42 $A_1B_1$ | 43 $A_1B_1$ | 44 $A_2B_1$ | 45 $A_2B_2$ | 46 $A_2B_1$ | 47 $A_1B_1$ | 48 $A_2B_1$ | 49 $A_{½}B_2$ | 50 $A_1B_1$ | 52 $A_2B_{½}$ |
| 36 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 37 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 38 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 39 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 40 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 41 $A_1B_1$ | n/a | - | - | - | + | - | - | - | - | - | - |
| 42 $A_1B_1$ | - | n/a | - | - | + | - | - | - | - | - | - |
| 43 $A_1B_1$ | - | - | n/a | - | + | - | - | - | - | - | - |
| 44 $A_2B_1$ | - | - | - | n/a | - | - | - | - | + | - | - |
| 45 $A_2B_2$ | + | + | + | - | n/a | - | + | - | - | + | - |
| 46 $A_2B_1$ | - | - | - | - | - | n/a | - | - | + | - | - |
| 47 $A_1B_1$ | - | - | - | - | + | - | n/a | - | - | - | - |
| 48 $A_2B_1$ | - | - | - | - | - | - | - | n/a | + | - | - |

*Note.* Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol. Only monokaryons from MBFBL 5 and monokaryon 52 from MBFBL 6 are shown in the horizontal axis of this table as these were the only monokaryons involved in successful interstrain or intrastrain crosses except for the monokaryons from MBFBL 2.

Table A4.1

*Cont.*

| Identification numbers and mating type of monokaryotic SSI cultures used in mating crosses | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SSIs | 41 $A_1B_1$ | 42 $A_1B_1$ | 43 $A_1B_1$ | 44 $A_2B_1$ | 45 $A_2B_2$ | 46 $A_2B_1$ | 47 $A_1B_1$ | 48 $A_2B_1$ | 49 $A_{½}B_2$ | 50 $A_1B_1$ | 52 $A_2B_{½}$ |
| 49 $A_{½}B_2$ | - | - | - | + | - | + | - | + | n/a | - | - |
| 50 $A_1B_1$ | - | - | - | - | + | - | - | - | - | n/a | - |
| 51 $A_2B_1$ | - | - | - | - | - | - | - | - | - | - | - |
| 52 $A_2B_{½}$ | - | - | - | - | - | - | - | - | + | - | n/a |
| 53 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 54 $A_2B_1$ | - | - | - | - | - | - | - | - | + | - | - |
| 55 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 56 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | - |
| 57 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | - |
| 58 $A_2B_1$ | - | - | - | - | - | - | - | - | - | - | - |
| 59 $A_1B_1$ | - | - | - | - | + | - | - | - | + | - | + |
| 60 $A_1B_1$ | - | - | - | - | + | - | - | - | - | - | + |

*Note.* Positive mating events are denoted by the + symbol and negative mating events are denoted by a – symbol. Only monokaryons from MBFBL 5 and monokaryon 52 from MBFBL 6 are shown in the horizontal axis of this table as these were the only monokaryons involved in successful interstrain or intrastrain crosses except for the monokaryons from MBFBL 2.

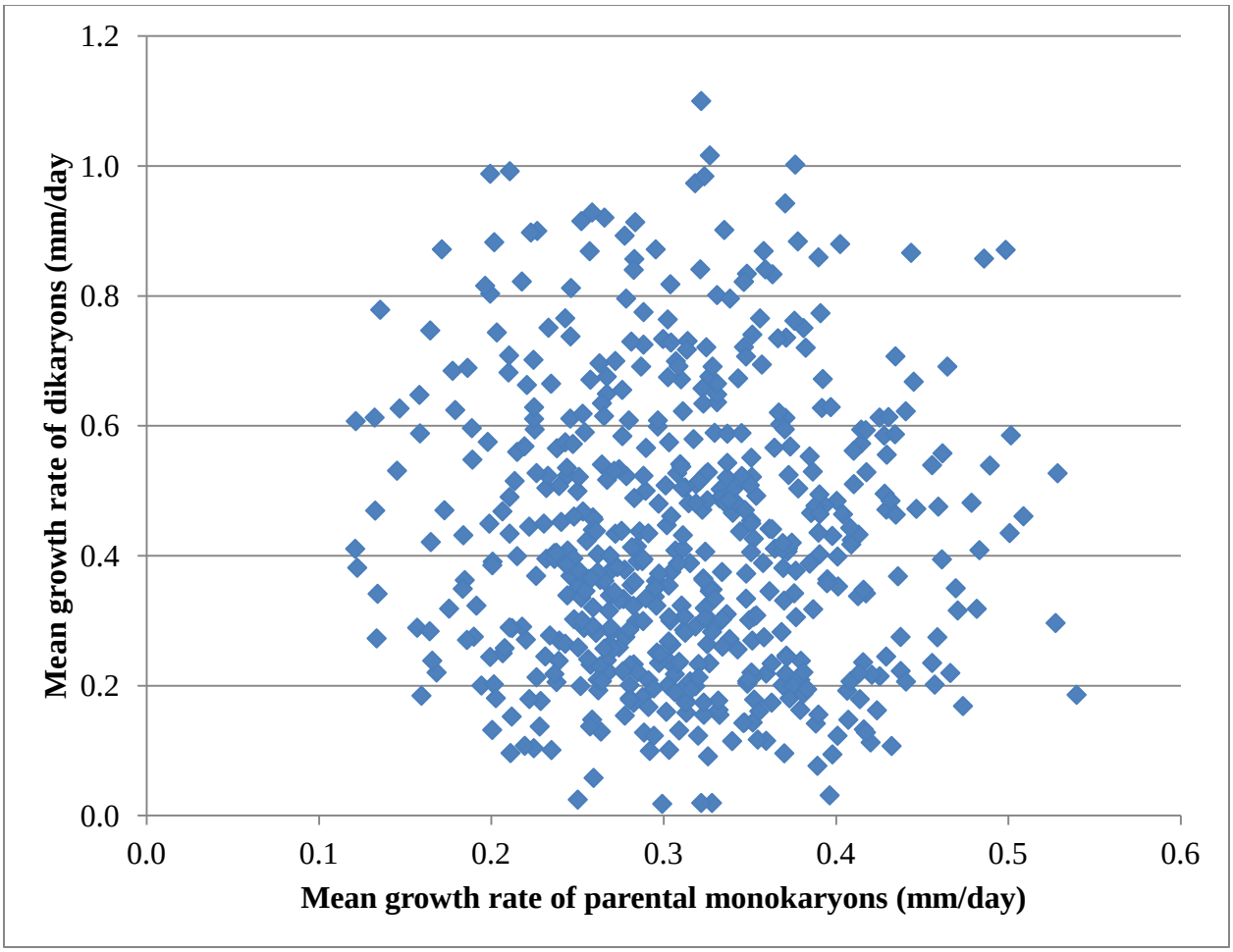


*Figure A4.1.* Plot showing lack of relationship between dikaryotic growth rate and the mean growth rate of constituent monokaryons at 10°C.

Table A4.2

*Analysis of variance for the effect of monokaryotic growth rate on dikaryotic growth rate at 10°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Regression | 1 | 0.03 | 0.03 | 0.70 | 0.4035 |
| Residual | 564 | 26.41 | 0.05 | | |
| Total | 565 | 26.44 | | | |

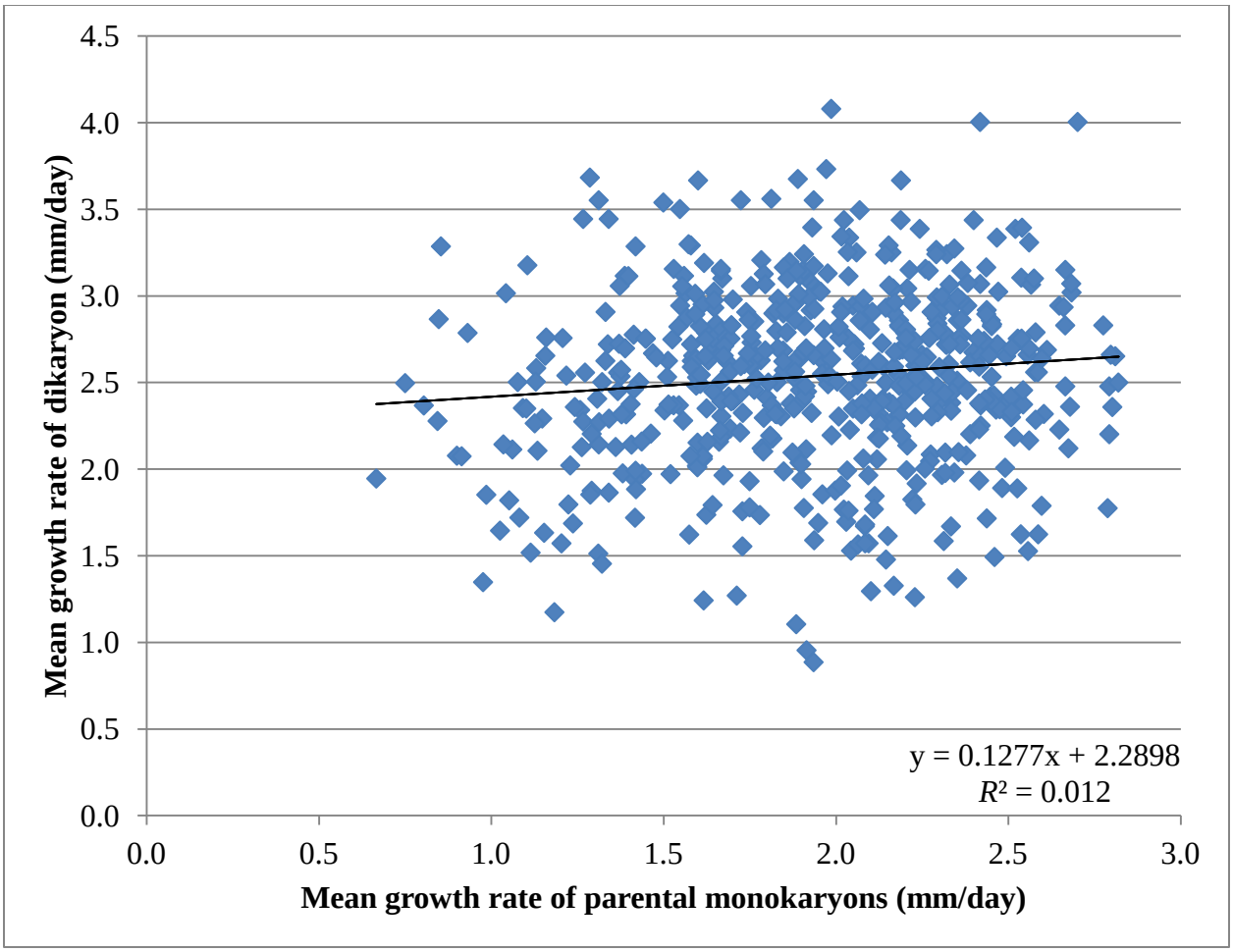


*Figure A4.2.* Relationship between dikaryotic growth rate and the mean growth rate of constituent monokaryons at 15°C.

Table A4.3

*Analysis of variance for the effect of monokaryotic growth rate on dikaryotic growth rate at 15°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Regression | 1 | 1.72 | 1.72 | 6.83 | 0.0092 |
| Residual | 564 | 141.77 | 0.25 | | |
| Total | 565 | 143.49 | | | |

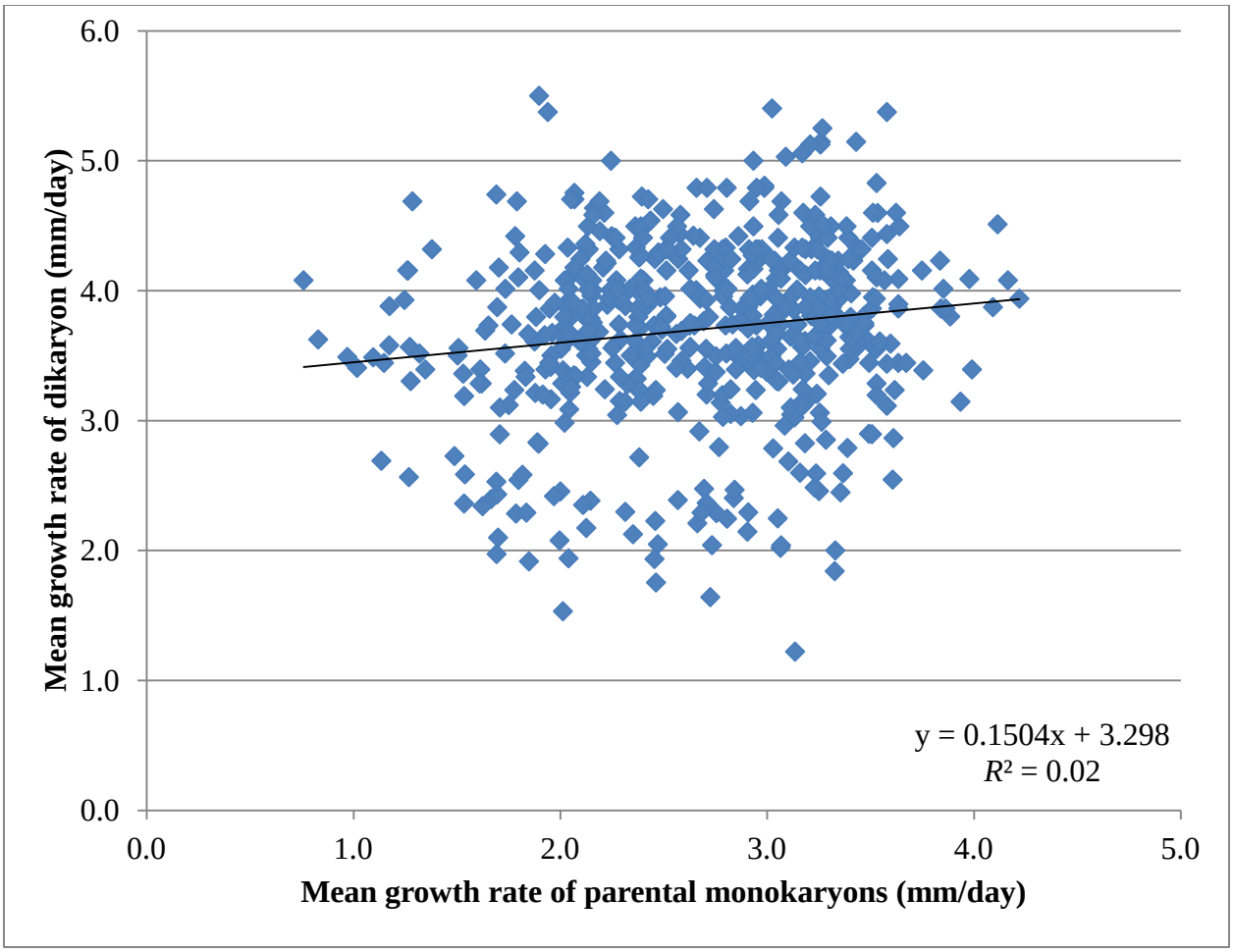


*Figure A4.3.* Relationship between dikaryotic growth rate and the mean growth rate of constituent monokaryons at 20°C.

Table A4.4

*Analysis of variance for the effect of monokaryotic growth rate on dikaryotic growth rate at 20°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Regression | 1 | 5.24 | 5.24 | 11.47 | 0.0008 |
| Residual | 564 | 257.88 | 0.46 | | |
| Total | 565 | 263.13 | | | |

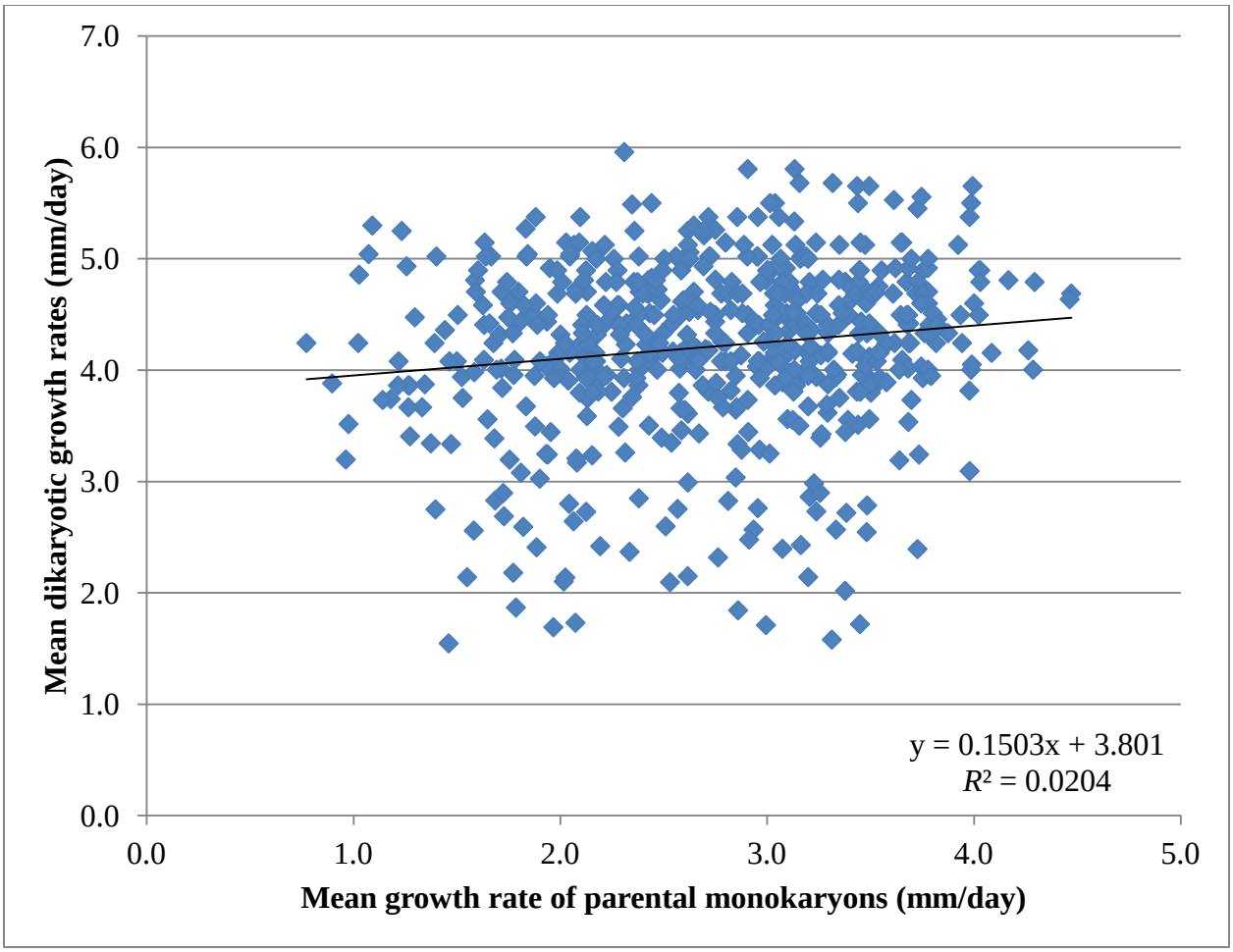


*Figure A4.4.* Relationship between dikaryotic growth rate and the mean growth rate of constituent monokaryons at 25°C.

Table A4.5

*Analysis of variance for the effect of monokaryotic growth rate on dikaryotic growth rate at 25°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Regression | 1 | 6.86 | 6.86 | 11.74 | 0.0007 |
| Residual | 564 | 329.45 | 0.58 | | |
| Total | 565 | 336.31 | | | |

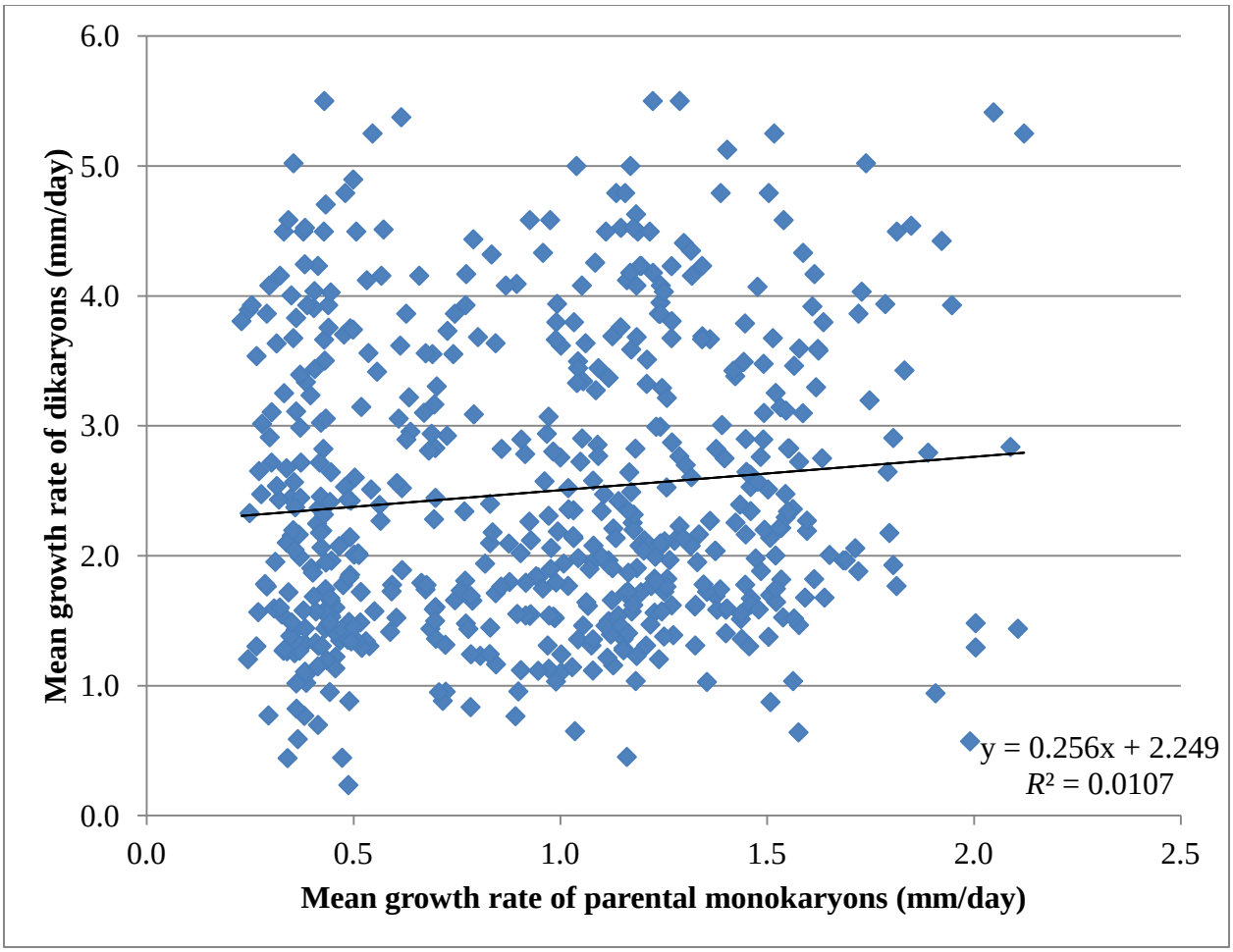


*Figure A4.5.* Relationship between dikaryotic growth rate and the mean growth rate of constituent monokaryons at 30°C.

Table A4.6

*Analysis of variance for the effect of monokaryotic growth rate on dikaryotic growth rate at 30°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Regression | 1 | 7.72 | 7.72 | 6.12 | 0.0137 |
| Residual | 564 | 712.13 | 1.26 | | |
| Total | 565 | 719.85 | | | |

*Appendix B*

Table B5.1

*ANOVA table for comparison of mycelial growth rates of parent strains at 10°C, 15°C, 20°C, 25°C and 30°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F* Value | Pr > F |
|---|---|---|---|---|---|
| Parent | 5 | 6.30 | 1.26 | 9.59 | <0.0001 |
| Temperature | 4 | 270.23 | 67.56 | 514.52 | <0.0001 |
| Parent x Temperature | 20 | 11.20 | 0.56 | 4.27 | <0.0001 |
| Error | 90 | 11.82 | 0.13 | | |
| Total | 119 | 299.55 | | | |

Table B5.2

*ANOVA table for comparison of mycelial growth rates of offspring from interstrain and intrastrain mating groups at 10°C, 15°C, 20°C, 25°C and 30°C*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | F Value | Pr > F |
|---|---|---|---|---|---|
| Full sibling groups | 11 | 537.84 | 48.89 | 98.25 | <0.0001 |
| Temperature | 4 | 19146.76 | 4786.69 | 9618.15 | <0.0001 |
| Mating cross x Temperature | 44 | 589.42 | 13.40 | 26.92 | <0 .0001 |
| Error | 11260 | 5603.79 | 0.50 | | |
| Total | 11319 | 25877.80 | | | |

Table B5.3

*Complete comparison of parent and offspring mycelial growth rates at 30°C.*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 1x2 | 4.08 | 2.00 | 0.0 | n/a | 32.0 | 1223, 1222, 1252, 1245, 1243, 1228, 1253, 1293, 1262, 1220, 1239, 1238, 1229, 1251, 1237, 1211, 1217, 1232, 1281, 1231, 1265, 1230, 1261, 1241, 1233, 1221, 1227, 1297, 1205, 1225, 1235, 1288 |
| 1x5 | 4.08 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 2x3 | 2.00 | 4.23 | 31.0 | 1366, 1369, 1563, 1362, 1361, 1526, 1359, 1344, 1568, 1341, 1365, 1537, 1356, 1370, 1558, 1345, 1338, 1551, 1353, 1327, 1540, 1348, 1364, 1558, 1328, 1339, 1578, 1347, 1360, 1552, 1367 | 0.0 | n/a |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Table B5.3

*Cont.*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 2x4 | 2.00 | 3.80 | 63.0 | 1443, 1424, 1444, 1446, 1431, 1483, 1426, 1432, 1496, 1436, 1433, 1445, 1456, 1438, 1434, 1442, 1452, 1430, 1506, 1486, 1439, 1435, 1466, 1450, 1441, 1447, 1440, 1468, 1490, 1505, 1455, 1420, 1448, 1484, 1416, 1464, 1460, 1461, 1425, 1423, 1493, 1494, 1453, 1502, 1428, 1415, 1421, 1495, 1508, 1457, 1411, 1454, 1437, 1449, 1451, 1498, 1418, 1485, 1462, 1414, 1463, 1491, 1422 | 10.0 | 1443, 1424, 1444, 1445, 1431, 1483, 1425, 1432, 1496, 1436 |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Table B5.3

*Cont.*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 2x5 | 2.00 | 3.62 | 61.0 | 1566, 1565, 1561, 1564, 1583, 1580, 1584, 1551, 1569, 1579, 1546, 1582, 1586, 1585, 1604, 1609, 1560, 1578, 1568, 1567, 1550, 1576, 1523, 1554, 1562, 1570, 1553, 1526, 1557, 1600, 1603, 1608, 1605, 1541, 1543, 1524, 1558, 1571, 1587, 1572, 1574, 1536, 1577, 1530, 1607, 1552, 1544, 1527, 1522, 1588, 1533, 1531, 1593, 1556, 1532, 1521, 1555, 1529, 1528, 1563, 1597 | 26.0 | 1566, 1565, 1561, 1564, 1583, 1580, 1584, 1551, 1569, 1579, 1546, 1582 1586, 1585, 1604, 1609, 1560, 1578, 1568, 1567, 1551, 1576, 1523, 1554, 1562, 1570 |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.

Table A5.3

*Cont.*

| Mating group | Average growth rate of Parent 1 (mm/day) | Average growth rate of Parent 2 (mm/day) | % of strains faster than parent 1 | MBFBL # of strains faster than parent 1 | % of strains faster than parent 2 | MBFBL #s of strains faster than parent 2 |
|---|---|---|---|---|---|---|
| 2x6 | 2.00 | 3.34 | 46.0 | 1694, 1681, 1691 1624, 1630, 1675 1703, 1699, 1625 1620, 1696, 1700 1662, 1610, 1674 1660, 1650, 1702 1688, 1671, 1670 1664, 1697, 1673 1695, 1704, 1690 1655, 1685, 1649 1684, 1698, 1640 1643, 1635, 1629 1663, 1619, 1652 1680, 1622, 1654 1644, 1659, 1683 1656 | 18.0 | 1694, 1681, 1691, 1624, 1630, 1675, 1703, 1699, 1625, 1621, 1696, 1700, 1662, 1610, 1674, 1660, 1650, 1702 |
| 3x5 | 4.23 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 4x5 | 3.80 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 5x6 | 3.62 | 3.34 | 0.0 | n/a | 0.0 | n/a |
| 2x2 | 2.00 | 2.00 | 0.0 | n/a | 0.0 | n/a |
| 5x5 | 3.62 | 3.62 | 0.0 | n/a | 0.0 | n/a |
| 6x6 | 3.34 | 3.34 | 0.0 | n/a | 0.0 | n/a |

*Note.* Strains found to have a greater growth rate are significant at the 5% level according to Duncan's multiple range tests.