BIOLOGICAL ASPECTS OF THE BIOPULPING FUNGUS *Ceriporiopsis subvermispora*

By

Ryan K. Goodale

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In Partial Fulfilment of the Requirements

for the Degree of Master of Science in Forestry

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BIOLOGICAL ASPECTS OF THE BIOPULPING FUNGUS *Ceriporiopsis subvermispora*

FACULTY OF FORESTRY AND THE FOREST ENVIRONMENT
LAKEHEAD UNIVERSITY
THUNDER BAY, ONTARIO

by

Ryan Goodale

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ABSTRACT

Goodale, R.K. 2002. Biological aspects of the biopulping fungus *C. subvermispora*.
Advisor: Dr. E. Setliff.

Key Words: white-rot fungi, cultural morphology, biopulping, growth rate, basidiocarp, basidiospores, sexuality, incompatibility, fungal growth, polyphenol oxidases.

The specific activity of four isolates of *C. subvermispora* media was found to be quite variable. Growth on PDA was the fastest compared to other common nutrient media. Cardinal temperatures for growth were optimum at 30°C, minimum at 15°C and maximum at 35°C. Macroscopic characteristics of mycelial mats, in relation to aerial hyphae production, were also found to differ among different media. Induction of fruiting was successful with two of the isolates when grown in petri dishes containing 1% malt extract agar. Basidiocarp production on *Pinus banksiana* boards was found to be very effective. Spore germination at 72% was highest with CZ-3 as was spore production. Single spore isolations and interfertility tests illustrated that *C. subvermispora* showed a tetrapolar incompatibility system and not a bipolar system as previously thought. The production of extracellular polyphenol oxidase in agar based media was found to be dependant on the type of media used.

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August 1, 2002
R. K. G.

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CHAPTER 1

GENERAL INTRODUCTION
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LITERATURE REVIEW

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INTRODUCTION

Ceriporiopsis subvermispora (Pil.) Gilbn. & Ryv. is a much studied fungus in the fields of biopulping and biopulping biochemistry. In spite of this high profile in forest biotechnological research, many aspects of its basic biology are unknown.

C. subvermispora is an uncommon saprophyte found in the Northern temperate regions that occurs on both gymnosperms and angiosperms (Gilbertson and Ryvarden 1993). In 1940, Pilát named the fungus *Poria subvermispora*, a member of the Polyporaceae. Lowe (1966) continued to maintain the fungus in *Poria*, but later Domanski (1969) proposed a change to *Fibuloporia subvermispora* (Pilát) Doman. Stalpers (1978) used this name in his descriptions of cultural characteristics of wood decomposers. However, Gilbertson and Ryarden (1985) re-classified the fungus to the genus *Ceriporiopsis*.

C. subvermispora is classified as a white-rot fungus (Gilbertson and Ryvarden 1987) and has the ability to completely break down lignin, a branched three-dimensional polymer that binds cells together and provide a protective coating around wood cells (Kirk and Cullen 1998). By comparison, brown rot fungi can only effectively decompose cellulose.

Mechanical and/or chemical methods are used to separate these wood cells (fibers) in the pulping process and both methods require large amounts of energy. Mechanical pulps are made by mechanically fiberizing the wood, while chemical processes are used to dissolve the lignin that binds the wood cells together. Chemical

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pulping results in pollutants of one kind or another, and a large percentage of the wood is dissolved and lost as wastewater (Kirk *et al.* 1993).

The concept of biopulping has been around for almost 50 years. Basically, the idea is to apply lignin degrading white-rot fungi to wood chips as a means of delignifying or softening wood prior to pulping (Kirk *et al.* 1993). It is only recently that headway is being made from the conversion of laboratory scale experiments to large-scale operations with industrial application (Akhtar *et al.* 1998). This study was undertaken with the goal of examining some aspects of the biology of *C. subvermispora*, thereby improving the biopulping process through greater understanding. The biological aspects of *C. subvermispora* examined were as follows: cultural characteristics (Chapter 2), development of basidiocarps and spore production (Chapter 3), test for determining incompatibility pattern (Chapter 4) and the production of extracellular polyphenol oxidase (Chapter 5).

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LITERATURE REVIEW

Relatively little research has been conducted on the life history of *C. subvermispora*. The main focus has been on biopulping research specifically biomechanical pulping (e.g. commercial application). Other studies include agro-based biopulping, biodegradation and biochemical pulping. As a consequence, numerous patents have been written on the use of *C. subvermispora* (Table 1.1) since its initial discovery as a good biopulping candidate in the 1970s (Setliff, personal communication Appendix I). Along with biopulping, ligninolytic enzyme systems have been under increasing study (Ruttimann *et al.* 1992; Ruttimann-Johnson *et al.* 1993; Lobos *et al.* 1994; Fukushima and Kirk 1995; Srebotnik *et al.* 1997; Larrondo *et al.* 2001). Although these studies deal mostly with ligninolytic systems, they provide some insight into the biology of the fungus. Table 1.2 shows the majority of biotechnological research completed over the past 20 years with *C. subvermispora.*

Various cultural studies have been made on *C. subvermispora* with isolates from both Europe and North America (Domanski 1969; Stalpers 1978; Nakasone 1981). In addition, morphological characteristics have been documented for taxonomical purposes (Pilát 1940; Lowe 1966, Domanski 1969; Gilbertson and Ryvarden 1987,1993).

A historical review of all research relevant to this study up to the present follows.

WHITE-ROT FUNGI

Not all white-rot fungi behave in the exactly the same way when it comes to wood decomposition. The two main systems now appreciated are either selective or

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simultaneous. Selective white-rotters consume lignin and hemicellulose prior to the degradation of cellulose. Conversely, simultaneous white-rotters uniformly degrade all

Table 1.1. Patents involving the use of *C. subvermispora* to 2001.

Patent Number	Year	Authors	Title
5055159	1991	Blanchette, R.A., G.F. Leatham, M. Attridge, M. Akhtar and G.C. Myers	Biomechanical pulping with *C. subvermispora.*
5460697	1995	Akhtar, M, M.C. Attridge, J.W. Koning and K.T. Kirk	Method of pulping wood chips with a fungi using sulfite salt-treated wood chips.
5472874	1995	Blanchette, R.A., R.L Farrell, and S. Iverson	Pitch degradation with white rot fungi.
5476788	1995	Lamar, R.T., D.M. Dietrich and J.A. Glaser	Solid phase bioremediation methods using lignin-degrading fungi.
5476790	1997	Blanchette, R.A., R.L Farrell, and S. Iverson	Pitch degradation with white rot fungi.
5620564	1997	Akhtar, M.	Method of enhancing biopulping efficacy.
5705383	1998	Blanchette, R.A., S. Iverson and C.J. Behrendt	Pitch and lignin degradation with white rot fungi.
5750005	1998	Akhtar, M	Method of enhancing biopulping efficacy.
5786188	1998	Lamar, R.T., D. Lestan, C.E. Smith and D.M. Dietrich	Fungal inoculum preparation.
5851351	1998	Baeker, A.A.W., and G.D. Shelver	Method of microbial pre-treating wood chips for paper making.
5865949	1999	Jaako, P., M. Siika-aho, and L Viikari	Process for preparing the treated mechanical pulp with an enzyme perparation having cellobiohydralase and endo- beta- glucanase activtity.
6099688	2000	Jaako, P., M. Siika-aho, and L. Viikari	Process for preparing mechanical pulp by treating the pulp with an enzyme having cellobiohydralase activity.
6143549	2000	Lamar, R.T., D. Lestan, C.E. Smith and D.M. Dietrich	Fungal inoculum preparation.
6267841	2001	Burton, S.W.	Low energy thermomechanical pulping process using an enzyme treatment between refining zones.

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cell wall constituents. Classification into these two systems differs among species and the level at which the systems operate differs among fungal isolates (Blanchette 1987; Blanchette 1991; and Blanchette 1995). Important in biopulping research are the white-rot fungi highly selective for lignin.

Table 1.2. Summary of literature studying fungal isolates of *C. subvermispora*.

Study	Isolate	Topic
Setliff and Eudy (1980)	FP-104027 L-6332	screening of white-rot fungi with high lignin specificity
Setliff *et al.* (1990)	L-6332	biomechanical pulping using Norway spruce, black spruce, white birch, trembling aspen and eucalyptus
Blanchette *et al.* (1991)	FP-90031	Biomechanical pulping U.S. Patent
Akhtar *et al.* (1992)	FP-90031-sp FP-105752 L-14807 L-15225 FP-104027	screening for most efficient strain for biomechanical pulping on loblloly pine chips
Blanchette *et al.* (1992)	CZ-3 ME-485 FP-90031-sp L-14807-sp L-15225-sp FP-104027-T L-3292-sp L-6133-sp	biopulping with white birch, trembling apsen and loblloly pine
Ruttimann *et al.* (1992)	L-14807 L-15225 FP-104027 FP-105752 FP-90031-sp	ligninolytic enzymes
Ruttimann-Johnson *et al.* (1993)	FP-105752 FP-90031-sp	enzyme production and synthetic lignin mineralization
Akhtar *et al.* (1993)	FP-90031-sp	biomechanical pulping of loblolly pine chips
Akhtar (1994)	CZ-3 L-6133 FP-90031-sp	biomechanical pulping of trembling apsen chips
Fischer *et al.* (1994)	CZ-3	resin reduction in biopulping

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Table 1.2 Continued...

Study	Isolate	Topic
Lobos *et al.* (1994)	FP-105752	Isoenzymes of MnP and laccase
Srenotnik *et al.* (1994)	FP-90031-sp	Lignin degradation study
Gamble *et al.* (1994)	FP-90031-sp	Biodegradation of Bermuda grass
Tapia and Vicuna (1995)	FP-105752	Lignolysis and pH
Fukushima and Kirk (1995)	CZ-3	Ligninolytic enzyme study
Jensen *et al.* (1996)	FP-90031	Lignolysis without lignin peroxidase
Rajakumar *et al.* (1996)	CZ-3	LiP-like genes in non-LiP producers
Blanchett *et al.* (1997)	CZ-3	Cell wall alterations in loblolly pine wood decay study
Srebotnik *et al.* (1997)	FP-90031-sp	Ligninolytic enzyme study
Akhtar *et al.* (1997)	CZ-3 L-14807 FP-105752	Corn steep liquor as an inoculum for biopulping
Urzua *et al.* (1998)	FP-105752	Products of MnP oxidation
Karahanian *et al.* (1998)	FP-105757	Structure and expression of the laccase gene
Galkin *et al.* (1998)	FP-90031-sp	Organic acids produced by different white-rot fungi
Temp *et al.* (1998)	L-6322-sp (8501)	Screening for ligninolytic fungi
Bustamante *et al.* (1999)	SS-3	Biomechanical pulping of bagasse
Mosai *et al.* (1999)	SS-3 CZ-3	Biosulfite pulping of eucalyptus
Enoki *et al.* (1999)	FP-90031	Extracellular lipid peroxidation
Chen *et al.* (1999)	FPL-14807-sp	Biopulping in compression-baled system for kraft and refiner pulping
Dorado *et al.* (1999)	FP-90031-sp	Delignification of wheat straw
Ramos *et al.* (2001)	SS-3	Biomechanical and biochemical pulping of sugarcane bagasse
Larrondo *et al.* (2001)	FP-105752	Isoenzymes of MnP

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OVERVIEW OF BIOPULPING STUDIES WITH *C. SUBVERMISPORA*

The development of biopulping research began nearly 50 years ago and was based upon knowledge of wood biodeterioration by fungal decomposers. Good indication that the biopulping process was feasible with white-rot fungi emerged in the mid-70s and continue up to the present (Duran *et al.* 1990; Kirk *et al.* 1992).

Current biopulping research have identified two white-rot species as being the most effective *viz., Phanerochaete chrysosporium* and *C. subvermispora* (Duran *et al.* 1990; Kirk *et al.* 1992; Messner 1998; Akhtar *et al.* 1998). The majority of the research is based on biomechanical processes, and the extent of delignification, energy savings, waste reduction, pulp yield and paper quality depends on the species and isolate used (Pilon *et al.* 1982; Blanchette *et al.* 1988; Setliff *et al.* 1990; Leatham *et al.* 1990; Blanchette 1991; Blanchette *et al.* 1992; Akhtar *et al.* 1992; Akhtar *et al.* 1993; Kirk *et al.* 1993; Fischer *et al.* 1994; Akhtar 1994).

Crowder *et al.* (1978) screened large numbers of white-rot fungi from North America and Africa specifically for their ability to degrade lignin at low weight losses. Two isolates of *C. subvermispora* (FP-104027 and L-6332), along with other fungal isolates, were found to be above average in being highly specific for lignin at early stages of decay and were fast growing (Setliff and Eudy 1980). Otjen *et al.* (1987) screened 30 white-rot fungi for their ability to selectively degrade lignin in wood blocks of *Pinus strobus* L. and *Betula papyrifera* Marsh., and *C. subvermispora* strain FP-90031-sp was found to be more effective on white birch wood blocks than white pine. Overall, *C. subvermispora* was a less effective lignin decayer than four other fungi.

One of the first published accounts of biomechanical pulping with *C. subvermispora* was by Setliff *et al.* (1990). *Populus tremuloides* Michx., *Betula*

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papyrifera Marsh., *Eucalyptus grandis* W. Hill ex Maiden, *Picea mariana* (Mill.) B.S.P, and *Picea abies* (L. ex Karst.) chips were treated with *C. subvermispora* isolate L-6332 and *P. chrysosporium* isolate ME-461. Overall, all species of chips showed signs of improved paper strength properties using *C. subvermispora* except for eucalyptus, which showed no sign of improved strength within the time frame used. The most significant energy savings came from treatments with *C. subvermispora* on aspen and Norway spruce chips.

The U.S. Biopulping Consortium

In 1987 a large program to advance biopulping research was established with the formation of a Biopulping Consortium in the United States. The research program was a collaboration of the U.S. Forest Service, Forest Products Laboratory, University of Wisconsin and the University of Minnesota, with financial contribution from nine forest industry companies. The number of companies in the consortium had grown to 22 by 2000. The overall goal of the research was to determine the industrial feasibility of biomechanical pulping. Achieving this goal required the transition of laboratory experiments to pilot scale operations (Kirk *et al.* 1992, Kirk *et al.* 1993, Akhtar *et al.* 2000). The following is a summary of the consortium's findings.

Nine isolates of *C. subvermispora* evaluated by Blanchette *et al.* (1992) showed little variation among their capacity to degrade lignin. Compared to *P. chrysosporium*, *C. subvermispora* was found to be better in lignin removal on the conifer *Pinus taeda* and the deciduous species *B. papyrifera* and *Populus tremuloides*. Isolate ME-485 degraded the highest percent of lignin in white birch (79.8%), L-3292-sp was top choice for loblolly pine (42.2%), and isolate CZ-3 was superior for trembling aspen (71.2%).

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Akhtar *et al.* (1992) screened five isolates of *C. subvermispora* for energy savings and paper strength properties for biomechanical pulping of *Pinus taeda*. Isolate FP-90031-sp produced the highest energy savings at 37%. Physical properties of handsheets were improved with all five fungal isolates compared to the control. Since the increase in strength properties was relatively similar between the isolates, FP-90031-sp was determined to be the most effective isolate. Akhtar *et al.* (1993) compared isolate FP-90031-sp to six other white-rot fungi in the biomechanical pulping of loblolly pine chips. Once again *C. subvermispora* proved to be superior to the other five fungi based on high pulp yields, energy savings and increased strength properties. The method of biomechanical pulping with *C. subvermispora* was granted a United States Patent (6267841) (Blanchette *et al.* 1991).

Akhtar (1994) studied the effects of *C. subvermispora* on *Populus tremuloides* pulp chips during the biomechanical pulping process with isolates, CZ-3, L-6133 and FP-90031. Out of the three isolates, CZ-3 saved the highest amount of electrical energy at 48% and produced the strongest physical handsheet properties. Isolate FP-90031 saved the least amount of energy (40%) and produced the smallest improvement in handsheet strength.

A key objective of the consortium was to develop a pilot scale biomechanical operation with industrial application. A project completed in 1998 developed a full-scale experiment that used *C. subvermispora* inoculum on 40 tons of aspen chips. The results of the project showed a 30% decrease in energy consumption and increased paper strength properties consistent with that of the laboratory studies. Additional results showed increased pulp yield and reduced effluents (Kirk *et al.* 1992, Kirk *et al.* 1993,

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Sykes 1994; Scott *et al.* 1998a,b; Akhtar *et al.* 2000). Economically the process was sound; however, production methods at the mill level still needed to be worked out.

The research group concentrated studies on methodologies for large-scale inoculation of chip piles (Kirk *et al.* 1993; Akhtar *et al.* 1997; Scott *et al.* 1998a,b; Jones *et al.* 1998; Akhtar *et al.* 2000). During the pilot-scale tests in 1998, a macerated mycelial suspension in corn steep liquor (Akhtar *et al.* 1997) was applied and mixed with the chips in a screw conveyer. A level of 5g of dry cut inoculum per tonne of chips was used in the process. Such an inoculum is inexpensive and produced satisfactory results (Akhtar *et al.* 1997; Akhtar *et al.* 2000). However, Kirk *et al.* (1993), showed a significant positive effect of inoculum level on energy savings for aspen treated chips with *C. subvermispora* in that increased levels of inoculum produced increased energy savings. For chips treated with *C. subvermispora* two types of inocula were tested: colonized chips and mycelium suspension. Both treatments were equally effective; however, spore suspensions were shown to be more effective. Aspen treated chips with *P. chrysosporium* using a conidiospore suspension proved to be more successful at reducing refiner energy in the pulp process. *P. chrysosporium* produces asexual conidiospores and chlamydospores regularly in culture. *C. subvermispora* only produces chlamydospores in culture (Gilbertson and Ryvarden 1987). Both fungi produce sexual spores called basidiospores; however, the cultural production of these spores has not been studies. Although conidiospores are asexual spores Kirk *et al.* 1993 stated the importance of spore production as an inoculum form,

> *P. chrysosporium* produces abundant conidiospores that have potential for use as a commercial inoculum. Unless methods for increasing sporulation of *C. subvermispora* are discovered, spore inoculum is not an option with this organism. The use of spores offers greater reproducibility and the ability to

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> accurately determine inoculum size. In addition spores have greater stability and are easier to package than other inoculum forms.

Akhtar *et al.* (1997) also commented on *C. subvermispora* as being a non-spore producer with the only source of inoculum being mycelial. Therefore, the use of spores as inoculum could open new doors to treating chips, for example in the use of belt conveyers as opposed to screw conveyers (Akhtar *et al.* 2000). Fungal spores are already being used commercially for reducing pitch content in chips. Cartapip™ is a packaged white powder of spores from the fungus *Ophiostoma piliferum* used in controlling pitch and resin problems in pulp mills (Farrell *et al.* 1992).

Agro-Based Biopulping

Delignification of wood and other materials with white-rot fungi has also become an attractive option in the agricultural industry for producing more carbohydrate-enriched products for ruminant animals (Dorado *et al.* 1999). Blanchette (1987) mentioned the use of rotted wood being used historically as forage by cattlemen in South America. Dorado *et al.* (1999) found *C. subvermispora*, isolate FP-90031, most effective in selective lignin degradation of straw as compared to *Pleurotus eryngii*, *P. chrysosporium*, and *Phlebia radiata*. Gamble *et al.* (1994) found the same isolate highly effective in converting Bermuda grass (*Cynodon dactylon* (L.) Pers.) into a more digestible form of animal feed.

In South America and Asian countries, the use of agro-based products such as bagasse in the pulping process is becoming increasingly attractive. Bagasse is the remaining fibrous residue of sugarcane after the juice has been extracted (Bustamante *et al.* 1999). Bustamante *et al.* (1999) and Ramos *et al.* (2001) used *C. subvermispora* in the biomechanical pulping of bagasse in order to improve the paper strength properties.

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Results showed highly increased paper strength and energy savings, with a minor loss of pulp yield and a decrease in paper brightness. In addition, Ramos *et al.* (2001) found similar results using pre-treatment of bagasse with *C. subvermispora* before chemithermomechanical pulping (CTMP).

Biodegradation

White-rot fungi are not only instrumental in biopulping, they are a key group in various aspects of biodegradation research (Koning 1988). Bioconversion of lignin-containing waste materials from pulps and urban and agricultural wastes has been researched for industrial application since the mid 1970's (Crowder *et al.* 1978). *C. subvermispora* has been examined in the reduction of resin (pitch) content in *Pinus taeda* L. (loblloly pine) and *Picea abies* (Norway spruce) wood chips. Fischer *et al.* (1994) found that *C. subvermispora* (isolate CZ-3) was just as effective at reducing resin content as the commercial product Cartapip™58. The commercial inoculum contains spores of *Ophiostoma piliferum* used by industry as means of reducing resin contents of wood chips in the pulping process. Overall, treatment with *C. subvermispora* produced increased paper strength and energy savings greater than that of *O. piliferum* and thus would seem to be a more appropriate fungus to commercialize.

Biochemical Pulping

A biochemical pulping process is one where the wood chips are first softened with white-rot fungi and then chemically pulped. There is little research on biochemical pulping with *C. subvermispora* compared to that of biomechanical pulping. Studies on biochemical pulping have only begun to appear over the past 10 years. Research has been concentrated on biomechanical pulping due to the many benefits of such a system.

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Though biochemical pulping is effective, it less attractive as a commercial option because of the lack of research compared to biomechanical pulping.

Applications of *C. subvermispora* in biosulfite pulping have been successful. Fischer *et al.* (1994) found isolate CZ-3 significantly reduced the kappa number and slightly increased paper strength with *Picea abies* chips. Mosai *et al.* (1999) compared the use of nine white-rot fungi on the biosulfite pulping of *Eucalyptus grandis*. *C. subvermispora* was able to reduce the kappa number of the pulp under that of the control and the other eight fungi. Two isolates of *C. subvermispora* were used, with SS-3 selected as the superior isolate over isolate CZ-3.

Chen *et al.* (1998) examined the effects of biokraft pulping with *C. subvermispora* on compression baled *Pinus banksiana* chips. Results indicated a 20% reduction in pulping time, and handsheet properties were similar to the control.

ENZYME RESEARCH

Another approach to biopulping is to use only the enzymes produced by fungi. Since 1990 biopulping research has shifted towards the study of ligninolytic enzyme systems. Overall, three main polyphenol oxidase enzymes have been identified and examined in the role of lignin degradation; *viz.* laccase, lignin peroxidase (LiP), and manganese dependent peroxidase (MnP) (Blanchette 1991; Fiechter 1991; Higuchi 1992; Messner 1998; Kirk and Cullen 1998). However, the literature indicates that some white-rot fungi, such as *C. subvermispora*, do not produce lignin peroxidase (Ruttimann *et al.* 1992a; Srebotnik *et al.* 1994). Theories as to the method of delignification by *C. subvermispora* have been developed, but the overall system has not yet been determined (Ruttimann *et al.* 1992a; Srebotnik *et al.* 1994; Tuor *et al.* 1995; Jensen *et al.* 1996; Rajakumar *et al.*1996; Srebotnik *et al.* 1997; Urzua *et al.* 1998; Enoki *et al.* 1999).

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Three newly discovered enzymes possibly involved in lignin degradation (aryl-alcohol oxidase, aryl-alcohol dehydrogenase and NADH: quinone oxidoreductases) could provide more insight into the process of delignification; however, these enzymes have yet to be isolated from *C. subvermispora* (Fiechter 1991; Leonowicz 1999).

When growing *C. subvermispora* on wood chips of *Pinus radiata*, the highest level of laccase was found during the first week of incubation. Lower levels were recorded in the second week. These levels over time were consistent with those found in liquid cultures; however, due to the unpredictable nature of laccase production, it is possible that this enzyme may be produced in older cultures (Lobos *et al.* 1994). On beech wood meal cultures inoculated with *C. subvermispora* FP-90031, laccase activity peaked on the third day of incubation with a second peak occurring on the 30th day (Enoki *et al.* 1999).

Fukushima and Kirk (1995) studied the specifics of laccase in lignin degradation. Their results indicated that laccase activity was affected by type of medium and pH levels with the best pH for laccase prodcution in the range of 3 to 5. Sodium azide and thioglycolic acid were the only two inhibitors of the enzyme identified in the study.

Growth conditions such as type of medium and pH also play a role in the production of manganese peroxidase. Larrondo *et al.* (2000) found growing media with less than 5% maltose affected the amount of MnP produced by *C. subvermispora.* Ruttimann-Johnson *et al.* (1993) and Tapia and Vicuna (1995) established that a medium containing 1% glucose produced higher levels of laccase and MnP, greater lignin mineralization, and better growth than cultures with 0.1% glucose. Furthermore, Ruttimann-Johnson *et al.* (1993) found low nitrogen levels increased lignin mineralization; however, MnP and laccase activity was highest at high nitrogen levels.

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Optimum pH for MnP activity was targeted at 4.5 with abrupt decreases in activity at pH 3.5 and 5.5 (Tapia and Vicuna 1995).

Although it seems that certain defined media and pH levels are key in producing the lignin-degrading enzymes of *C. subvermispora*, it is only in the natural environment where the complete ligninolytic system can be observed. Comparing the lignin degrading mechanisms of LiP producer *P. chrysosporium* and non-LiP producer *C. subvermispora*, Srebotnik *et al.* (1994) found that *P. chrysosporium* had more efficient lignin degrading mechanisms than *C. subvermispora* when growing on certain defined media. However, on wood blocks of *Betula papyrifera*, *C. subvermispora* degraded lignin just as rapidly and completely; thereby indicating certain mechanisms of the lignolytic system are apparently absent on some defined media.

Other influences on enzyme activity, such as aeration, have been noted. Ruttimann *et al.* (1992a) found the exposure of *Phlebia brevispora* cultures to oxygen stimulated enzyme production. Ruttimann-Johnson *et al.* (1993) showed the daily exposure of *C. subvermispora* cultures to oxygen slightly increased lignin mineralization, yet MnP and laccase levels remained unchanged.

More recently, Galkin *et al.* (1998) have shifted toward examining organic acids produced by white-rot fungi. This study suggested that organic acids such as oxalic acid may play a key role in lignin degradation. *C. subvermispora* isolate FP-90031-sp was found to produce the highest levels of oxalic acid compared to 15 other white-rot fungi. Oxalates were also observed by Urzua *et al.* (1998). Furthermore, Kirk *et al.* (1993) and Akhtar *et al.* (1998) observed that *C. subvermispora* readily produced calcium oxalate crystals when growing on wood chips using SEM technology.

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CULTURAL CHARACTERISTICS

Although many white-rot fungi have been studied for cultural characteristics (Davidson *et al.* 1942; Nobles 1958b; Stalpers 1978; Wang and Zabel 1990), there is little known about the natural life history of *C. subvermispora* (Gilbertson and Ryvarden 1993). The cultural characteristics in this paper will follow the methods formulated by Davidson *et al.* (1942), Nobles (1948, 1958b, 1965), Stalpers (1978) and Wang and Zabel (1990). Primarily, there are five major features observed for cultural characterization: presence/absence of extracellular polyphenol oxidase, sexuality, growth characteristics, and macroscopic and microscopic features of hyphae and special structures (Nobles 1948, 1958b, 1965; Stalpers 1978). In addition, fruiting body production will be examined in culture.

Tests for extracellular oxidase

The polyphenol enzymes laccase and tyrosinase are extracellular oxidase enzymes, meaning that the enzymes function outside the hyphal walls and are oxidative (requires O_2). Extracellular oxidases are produced by the majority of all white-rot fungi and are used to distinguish against the brown-rot fungi which fail to produce these enzymes (Nobles 1958a; Nobles 1958b; Ruttimann-Johnson *et al.* 1993; Tuor *et al.* 1995). There have been many tests developed for identifying the presence of extracellular oxidase in growing cultures (Bavendamm 1928; Nobles 1958a; Harkin *et al.* 1974; Stalpers 1978; Marr 1979; Rayner and Boddy 1988). One such test developed by Nobles (1958a) proved to be fairly accurate and quick for identifying cultures. Nobles used an alcoholic gum guaiac solution on 113 species of white-and brown rot Hymenomycetes against the more popular gallic and tannic acid Bavendamm test for polyphenol oxidases (Bavendamm 1928). All 113 species were grown on a malt agar

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medium (percent of malt not given) and after 2 weeks of growth, a drop of solution was placed on the actively growing mycelium. The laccase reaction was found to be very quick in that a blue colouration appeared after 2-3 minutes. Results indicated that the test was 85% in agreement with the Bavendamm method. Other cultures gave inconsistent results. Nobles (1958b; 1965) continued using the gum guaiac test for laccase as a taxonomic method to separate between brown and white-rot fungi in the cultural classification of the Polyporaceae. Although quite effective, the Bavendamm method requires a period of 1-week to produce a discernible reaction (Rayner and Boddy 1988). Stalpers (1978) used α-naphthol to test for laccase, *p*-cresol for tyrosinase and pyrogallol + hydrogen peroxide for peroxidase. Such tests, although relatively consistent were read after 3, 24 and 72 hours. Marr (1979) found that the gum guaiac test did not accurately distinguish between laccase and tyrosinase. Syringaldazine is a specific test used for identifying laccase but has yet to be incorporated into cultural studies (Harkin *et al.* 1974). I adopted Nobles' (1958a) gum guaiac test for my research in an effort to understand why some white-rot fungi (15%) fail to react positively to it in a short period of time.

Sexuality

According to Gilbertson and Ryvarden (1987, 1993), the cultural characteristics and sexuality pattern of *C. subvermispora* are unknown. However, Nobles *et al.* (1957), Stalpers (1978), Domanski (1969) and (Nakasone 1981) gave accounts of cultural characteristics and established the sexuality or incompatibility system as being heterothallic bipolar. Recent work done by Hibbett and Donoghue (2001), using phylogenetic rDNA analysis and ancestral state reconstruction, also suggest that the fungus is bipolar.

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There has been extensive work done on determining the sexuality of Basidiomycetes, although most are still unknown. The work of Aschan (1954), Aschan-Aberg (1960a,b), Takamaru (1961), Raper (1966a,b), Esser (1966), Setliff (1970), Ginns (1974) and Casselton and Kues (1994) have developed important analytical methods in determining fungal sexuality based upon clamp connection formation. Methods detailed in these studies will be used as a guide in determining the sexuality of *C. subvermispora*.

Growth and hyphal characterisitics

Gilbertson and Ryvarden (1987, 1993), Lowe (1966), Stalpers (1978), Domanski (1969) and Pilát (1940) have published morphological observations of *C. subvermispora* basidiocarps. Domanski (1969), Stalpers (1978) and Nakasone (1981) provided descriptions of this fungus in culture (see Table 1.3). Domanski (1969) provided detailed cultural descriptions for the presumptive haploid and dikaryotic hyphae of *C. subvermispora*, but failed to determine cardinal growth. Stalpers (1978) gave some insight as to the growth characteristics of *C. subvermispora*. He examined the margin of growth, the area of actively growing mycelium, and aerial hyphal characteristics. However, the cardinal temperatures for growth were undetermined which is one of the characteristics used by Nobles (1965). Nakasone (1981) published the most detailed cultural account of the fungus identifying the growth characteristics, microscopic characteristics and incompatibility system of the fungus; however, the temperature range for growth was undetermined. Biopulping studies have used various temperatures and media for optimum growth rates as shown in Table 1.4. Scott *et al.* (1998a) reported that the optimum growth temperature for *C. subvermispora* was somewhere between 27 and 32°C with PDA (potatoe dextrose agar) as being the choice medium, and other

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Table 1.3. Summary of cultural characteristics of *C. subvermispora* by Domanski (1969), Stalpers (1978) and Nakasone (1981).

	Domanski (1969)	Stalpers (1978)	Nakasone (1981)
Nomenclature	*Fibuloporia subvermispora* (Pilát) Doman.	*Fibuloporia subvermispora*	*Poria subvermispora* Pilát
Interfertility	Bipolar	Heterothallic Bipolar	Bipolar
Extracellular oxidase	Not reported	Laccase +, Tyrosinase +, Peroxidase +	Gallic acid +, Tannic acid +
KOH	Not reported	negative	Not reported
Growth Rate	Not reported	over 70mm in 7-14 days	Rapid, plates covered in 1 week
Marginal hyphae (of advancing zone)	appressed, translucent, with numerous irregular clusters of white cottony mycelium. Hyphae rarely septate, 2.5-7μm wide	appressed or submerged with distantly spaced hyphal tips	margin even, appressed, thin-walled, simple septate, infrequently branched, 6-7μm
Aerial mycelium	loosely cottony	downy, farinaceous, granulose, cottony	subfelty to felty, raised or woolly, sometimes tufted
Mycelial mat	whitish	white	white
Hyphae	strongly branched, 2-3.5μm thick, generative hyphae thin walled, seldom thickened walls	variable branching, 1.5-7.5 μm in width, sometimes encrusted, variable swellings	branched, 2-4μm, thin-walled or slight wall thickening,
Clamps	nodose-septate	rare to inconsistently occurring at all septa	nodose septate
Chlamydospores	abundant spherical or pear shaped , terminal and intercalary, 8.5-17.5 x 7-12.5μm	present, 8-18 x 7-14 μm	globose to limoniform, 11.5-13.5(-16) x 7.5-13.5 (-16) μm, thin-walled, walls thickening in age, hyaline, terminal or intercalary, numerous in submerged and aerial mycelium.
Crystals	hyphae encrusted by spherical aggregates of crystaline bodies	in aerial mycelium and agar	aerial hyphae occasionally encrusted with hyaline crystals

studies confirms these findings (Table 1.4). Furthermore, Scott *et al.* (1998a) found 42°C was too high for the survival of the fungus in chip piles. Srebotnik *et al.* (1997) observed that *C. subvermispora* is not viable at 39°C on PDA medium.

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Growth rates are established differently by many authors because there is no single standard for describing cultures. Nobles (1948, 1965, 1958b) used 1.25% malt extract with 2% agar at room temperature with a minimal exposure to light. Growth rates were determined by recording the number of weeks required for the hyphae to extend across the agar surface. Stalpers (1978) determined growth rates on 2% malt extract with 1.9% agar medium at 17-20°C under diffuse daylight. Growth was measured by the radial extension of the hyphae after 1- and 2-week time periods. Wang and Zabel (1990) measured radial extension at 1- and 2-week time periods to establish growth rates of fungal cultures growing at 25°C on a medium containing 1.5% malt extract and 2% agar.

Table 1.4. Examples of optimum temperatures and media used for growing *C. subvermispora* in culture by various authors.

Author	Temperature	Medium*
Nakasone (1981)	32°C	MEA
Setliff *et al.* (1990)	30°C	MEA
Blanchette *et al.* (1992)	27°C	-
Kirk *et al.* (1993)	27°C	PDA
Akhtar (1994)	27+/-1°C	PDA
Srebotnik *et al.* (1994)	29°C	PDA
Lobos *et al.* (1994)	30°C	PDA
Tapia and Vicuna (1995)	30°C	-
Fischer *et al.*(1994)	27°C	PDA
Akhtar *et al.* (1997)	27+/-1°C	PDA
Urzua *et al.* (1998)	30°C	PDA
Bustamante *et al.* (1999)	27°C	PDA
Mosai *et al.* (1999)	29+/-1°C	PDA

*MEA = malt extract agar, PDA = potato dextrose agar

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Fruiting

Many white-rot fungi have been documented as unlikely to fruit in culture (Nobles 1948; Wang and Zabel 1990; Croan 1994), and *C. subvermispora* was considered to be in this category. Rayner and Boddy (1988) outlined four main external factors influencing fruiting, *viz*. substrate, light, air and temperature. They showed that many substrates or defined media must be used under a variety of conditions in order to induce fruiting. Factors such as the pH in a medium may play a key role in basidiocarp formation. Wessels (1994) suggested light had variable effects on fungal fruiting. Some did not require any light while others did. Aeration and humidity levels are major influences in fruiting. Generally, high oxygen and low carbon dioxide levels have positive effects on fruiting of some basidiomycetes (Wessels 1994; Rayner and Boddy 1988).

Similar to growth rates, fruiting body development must have cardinal temperatures that govern the initiation of basidiocarp formation and basidiospore production; however, once a basidiocarp is formed sporulation becomes an additional complex system to predict and control (Kramer 1982).

Although this paper only deals with the external factors affecting fruiting, it should be noted that the internal factors of the fungus are of major importance. Rayner and Boddy (1998) and Wessels (1994) gave a brief explanation of such factors and their roles in sporocarp formation.

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CHAPTER 2

CULTURAL CHARACTERISITICS

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INTRODUCTION

Cultural studies of most basidiomycetes typically examine growth characteristics, macroscopic features, microscopic features, sexuality, and extracellular oxidase reactions (Nobles 1965; Stalpers 1978; Rayner and Boddy 1988; Wang and Zabel 1990). This chapter will examine the cardinal temperatures for growth, macroscopic characteristics and microscopic characteristics of *C.subvermispora.*

In many cultural studies growth rates are determined by examining the radial growth of fungi on a weekly basis or by recording the amount of time it takes to grow across a petri dish (Nobles 1948; Stalpers 1978; Gilbertson and Ryvarden 1986; Wang and Zabel 1990). When examining the growth rates at various temperatures, the cardinal points of temperature can be identified. The maximum, minimum and optimal temperatures for growth are another widely used cultural attribute (Rayner and Boddy 1988). Although the literature suggests an optimum temperature range for *C. subvermispora* (see Table 1.4), the low and high cardinal points have yet to be determined. Such information could provide useful information in biopulping research regarding the growth rate and survival of this fungus in various aspects of the chip piles.

The appearance of mycelial mats can be a significant tool in identifying and classifying fungi grown in culture (Davidson *et al.* 1942; Nobles 1948; Nobles 1958b; Stalpers 1978; Wang and Zabel 1990). Macroscopic features of the mycelial mat such as texture, colour and pattern of growth are described in specific terms to describe their visual appearances. Terms used by Nobles (1948, 1965), Stalpers (1978), Gilbertson

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and Ryvarden (1986), and Wang and Zabel (1990) will be adopted to describe the characteristics of *C. subvermispora.*

Microscopic features of hyphae and special structures are by far the most important for identifying fungi in culture. The identification of microscopic features of *C. subvermispora* and the terminology used to describe these features will follow the work of Davidson *et al.* (1942), Nobles (1948, 1958b, 1965), Lombard and Gilbertson (1965), Stalpers (1978), Lombard (1983), Gilbertson and Ryvarden (1986), and Wang and Zabel (1990).

Visual and microscopic characterization of fungal cultures is typically based on one growing medium. The most common media used to grow basidiomycetes in culture are malt extract agar (MEA) and potato dextrose agar (PDA) (Gilbertson and Ryvarden 1986; Rayner and Boddy 1988; Wang and Zabel 1990). This chapter will examine the effect of six different media on the macroscopic features of two isolates of *C. subvermispora.*

The purpose of this chapter is to identify the cultural features and growth characteristics of *C. subvermispora.* The fungal isolates and media used within this chapter are identified in the materials and methods section. Materials, methods and results for: estimating pH levels of the media, cardinal temperatures for growth, macroscopic and microscopic features of hyphae are presented. A discussion on the results of the four experiments follows.

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MATERIALS AND METHODS

Fungal Isolates

Four isolates of *C. subvermispora* were obtained from the Center for Forest Mycology Research in Madison, WI and maintained on slants and petri dishes of 1% Difco malt extract agar (MEA) at 27°C (Table 2.1).

Table 2.1. Sources of *C. subvermispora* isolates.

Isolates	Host	Location	Reference
L-6332	*Pinus contorta* var. *latifolia*	Roosevelt National Forest, CO, July 1963	Collected and determined by J.L. Lowe. Center for Forest Mycology Research, FPL, Madison, WI. (Setliff *et al.* 1990)
CZ-3	*Tsuga heterophylla* (Raf.) Sarg.	Port Townsend, WA	Center for Forest Mycology Research, FPL, Madison, WI. (Blanchette *et al.* 1992)
ME-485	*Pseudotsuga menziesii* (Mirb.) Franco	Wauna, OR	Center for Forest Mycology Research, FPL, Madison, WI. (Blanchette *et al.* 1992)
FP-90031-sp	Oak house log	Beltsville, MD	Isolated by R.W. Davidson, R.L. Gilbertson, and Ed Haschaylo. Center for Forest Mycology Research, FPL, Madison, WI. (Blanchette *et al.* 1992; Kirk *et al.* 1993)

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Media

Six nutrient media were used for a variety of experiments in this chapter and in the following chapters to examine cultural characteristics, fruiting and extracellular enzyme production (Table 2.2). The choice of media was determined from what was generally recommended by the literature. The Norway spruce medium was formulated to more closely emulate the natural substrate and nutrient levels available to the fungus.

Table 2.2. Media used for various experiments.

Medium	Acronym	Nutrient*	Percent Agar
1% Malt Extract Agar	MA1%	1% Malt Extract	1.5
3% Malt Extract Agar	MA3%	3% Malt Extract	1.5
Potato Dextrose Agar	PDA	200g/L of potato infusion 2% Bacto-dextrose	1.5**
Corn Syrup Agar	CS0.15	0.5% Corn syrup 0.15% Glucose	1.5
Corn Syrup/Yeast Extract	CSY	0.5% Corn syrup 0.1% Bacto yeast extract	1.5
Norway Spruce Infusion	NSA0.15 NSA0.30 etc.	0.15%, 0.30%, etc. glucose Norway spruce infusion	1.5

*all natural media are Difco™ products
**Bacto-Agar, all others contain granulated agar.
Corn syrup = Lily White®

The methodology for producing the Norway spruce infusion agar consisted of taking: 800cc of Norway spruce chips and adding them to a beaker with 1 L of water. The chips were then boiled for 20 minutes. The appropriate percent age of glucose and

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agar was added to the pale yellow broth. Norway spruce agar used for Chapter 5 consisted of pure Norway spruce infusion agar at four different concentrations of glucose: 0.15%, 0.3%, 0.6% and 1.2%. The media were autoclaved for 20 minutes at 121°C and 20psi. Plastic petri dishes of size 8.8 x 1.5 cm were used for all experiments.

Determining pH levels of media

Because pH can impact basidiocarp formation (Rayner and Boddy 1988) and the production of extracellular oxidases (Marr 1979; Fukushima and Kirk 1995; Tapia and Vicuna 1995), the pH of the six different media was determined. Two other media were also tested, NSA (Norway spruce infusion agar) at 1.2 and 0 percent glucose, to determine the influence of high and to nil concentrations of sugar on fruiting with *C. subvermispora.*

Assuming that the media would fall in the range of pH 5 or 6, a digital pH meter was calibrated at a pH of 4.0 and then at 7.0 using Canlab™ reference buffer solution. The reference buffer solutions were heated to 60°C since the media had to remain heated at this temperature in order to remain liquid. Calibration between the reference solutions resulted in a discrepancy of pH 0.5. Thus, all pH values were rounded to the nearest 0.5 unit to establish an estimated pH level. Each medium was measured three times and averaged, to the nearest 0.5.

Cardinal temperatures for growth

Using one growth chamber, isolate CZ-3 was grown at seven different temperatures: 0°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C. A medium of 1.5% malt extract with 2% agar was used as suggested by Davidson *et al.* (1942) and Wang and

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Zabel (1990). Four petri dishes were inoculated at one edge with a 0.4mm mycelial plug for each temperature. Radial growth was measured at 1-and 2-week intervals.

Macroscopic Features of cultures

The growth patterns of mycelia in culture on the six different media were photographed with a 35mm camera for isolates CZ-3 and L-6332. Three plates of each medium were cultured and the most consistent visual pattern was photographed. The cultures were 1+ month old when photographed. Observations for aerial hyphae, texture, colour and appearance of the mycelial mats for each medium were documented on a daily basis during the growth experiments.

Microscopic analysis of cultures

Cultures from isolate CZ-3 and L-6332 were grown on 1% malt extract and examined microscopically. Hyphae and special structures such as chlamydospores were observed microscopically and photographed. Young hyphae in the advancing edge of mycelial growth were observed after 5 days and mature hyphae after 1-month of growth.

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RESULTS

Determining pH levels of media

As shown below, pH values by media varied by 2.5 pH units between the highest and lowest pH averages (Table 2.5).

Table 2.3. Average pH for eight growing media.

Medium	pH
MA3% (3% malt extract, 1.5 % agar)	5.5*
MA1% (1% malt extract, 1.5% agar)	7.5
PDA (potato dextrose agar)	5.0*
CS0.15 (0.5% corn syrup, 0.15% glucose, 1.5% agar)	7.0
CSY (0.5% corn syrup, 0.1% yeast extract, 1.5% agar)	7.0
NSA0.15 (Norway spruce infusion, 0.15% glucose, 1.5 % agar)	6.5
NSA1.2 (Norway spruce infusion, 1.2% glucose, 1.5 % agar)	6.5
NSA (Norway spruce infusion, 1.5% agar)	6.5

* Difco™ indicates that its 3% malt extract agar has a pH of 5.5±0.2. However, the PDA was found to have a pH of 5.0±.5 rather than pH 5.6 indicated by the manufacturer.

PDA and MA3% had the most acidic pH out of all eight media. The three glucose levels used for the NSA medium proved to have no impact on the pH of the medium, all three values were slightly acidic at a pH of 6.5. Both corn syrup based media were neutral at 7.0, while MA1% was slightly basic at a pH of 7.5.

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Cardinal temperatures for growth

Figure 2.1 plots the growth rates of isolate CZ-3 on 1.5% malt extract agar over 1-and 2-week periods for seven different temperatures. The data that was recorded is presented in Appendix I Table AI.1. In the first week no growth occurred at 10, 15 and 40°C and the optimum growth rate occurred at 30°C. In the second week, no growth was observed at 10°C and 40°C; however, there was some growth at 15°C. At temperatures of 25°C and 30°C the mycelia extended the diameter of the petri dishes at 8.8 cm before the end of two weeks. At 25°C the mycelium radially extended across the petri dishes after 13 days, while the dishes at 30°C were covered after only 9 days.

Macroscopic Features of Cultures

Each media had an effect on growth patterns, colour, texture, and formation of special structures. The richer media had thicker, whiter mycelial mats because of a proliferation of aerial hyphae. Terms used to describe the textures and patterns of growth for isolates CZ-3 and L-6332 on each medium follows the work of Stalpers (1978).

Figures 2.2 and 2.3 show the mycelial mats of CZ-3 and L-6332 grown on CS 0.15%. Both isolates produced appressed hyphae in a feathery fan-like pattern radiating from the inoculation point, this pattern seemed to be more distinct with isolate L-6332. Aerial hyphae formed around the inoculation point exhibiting sparse white tufts after six days of growth. Tufts continued to emerge around the central axis as the mycelium covered the dish. Medium discolouration was not observed after a month of growth.

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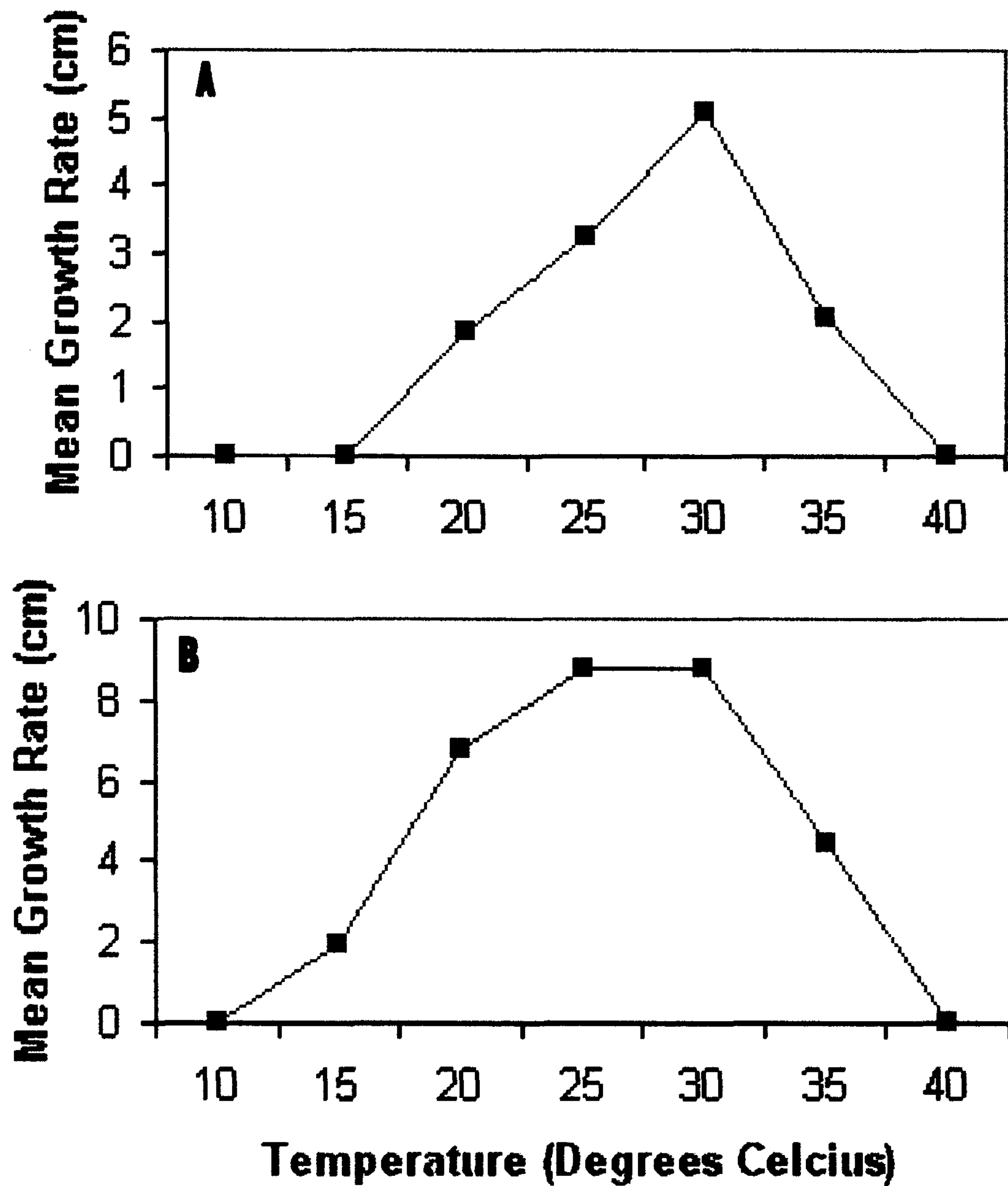

Figure 2.1. Cardinal temperatures for isolate CZ-3 grown on 1.5% malt extract agar after 1week (A) and 2 weeks (B).

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A distinct growth pattern was observed on MA3% plates inoculated with isolate CZ-3. Aerial mycelium was arranged in a plumose design resembling bird feathers (see Figure 2.4). This pattern was present during the advancing stages of mycelium from L-6332 but became less distinct over time. After a month of growth, L-6332 developed a floccose appearance of small hyphal tufts scattered across the surface of the medium resembling a grainy texture (see Figure 2.5). The medium was slightly bleached by the fungus; however, a malt colouration was still present after one month.

Visible differences in texture and pattern occurred at the lower nutrient levels of malt. MA1% produced a faint fan-like pattern during hyphal advancement, aerial hyphae developed after a week of growth arranging in white floccose patches. Isolate CZ-3 had been observed to produce thicker and whiter patches of aerial hyphae compared to L-6332 (see Figure 2.6 and 2.7). Complete bleaching of the medium usually occurred after two weeks but the fungus had been noted to take much longer.

The CSY medium resembled the growth patterns on CS0.15%. Isolate CZ-3 developed a distinct fan-like pattern of appressed long and short hyphal groupings (see Figure 2.8). In comparison, L-6332 had a less distinct arrangement that appeared as fine hairs of appressed hyphae that radiated from the central axis (see Figure 2.9). Aerial hyphae were only observed in small sparse groupings around the inoculation point for both strains. The overall colour of the mat was translucent due to the large mat of appressed hyphae. No bleaching effect in the medium was observed.

PDA produced a floccose texture consisting of aerial hyphae grouped in erect white tufts protruding out from the agar surface. The tufts appeared after 6 days of growth and continued to develop with age. A grainy appearance was produced due to the large amount of tufts scattered over the entire surface of the medium. The tufts are

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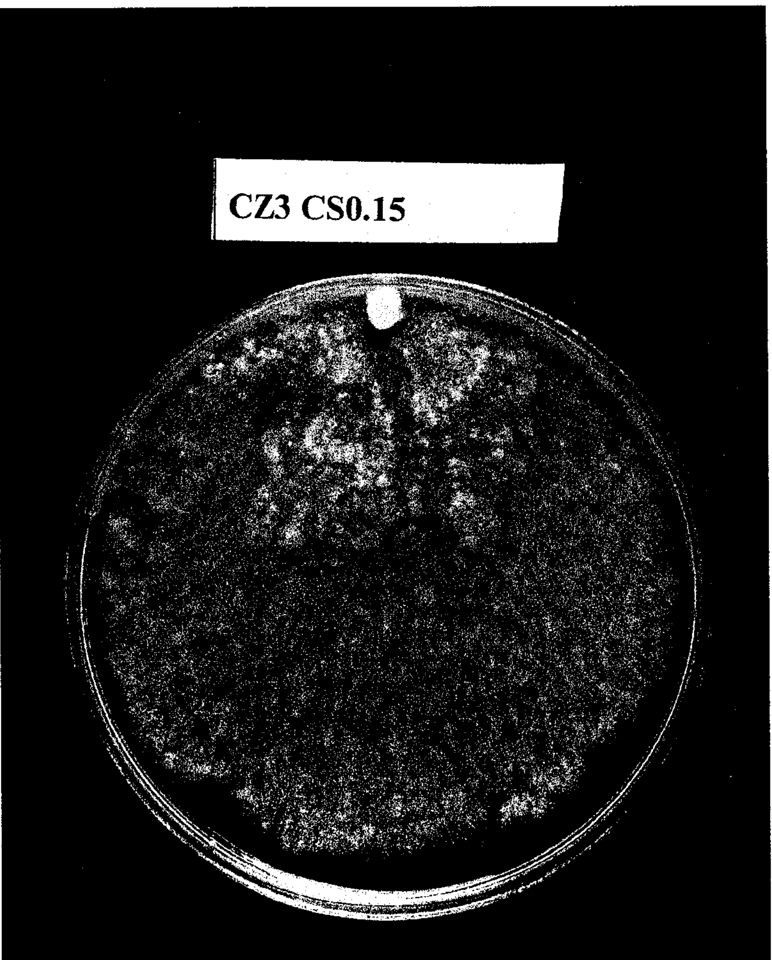

Figure 2.2. Growth of isolate CZ-3 on CS0.15.

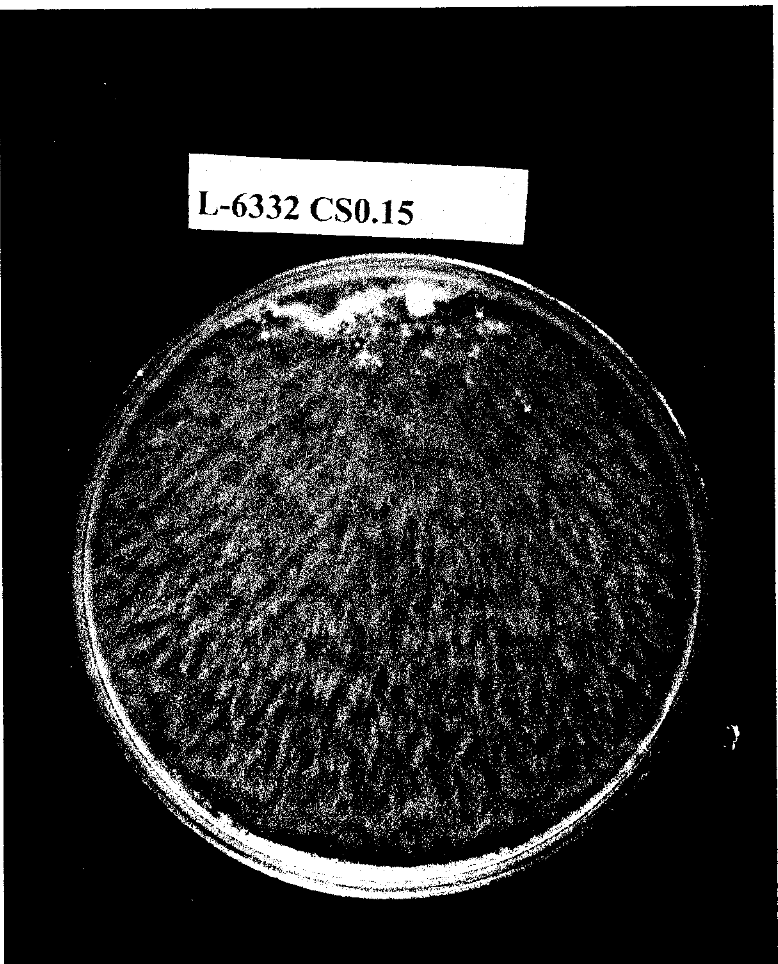

Figure 2.3. Growth of isolate L-6332 on CS0.15.

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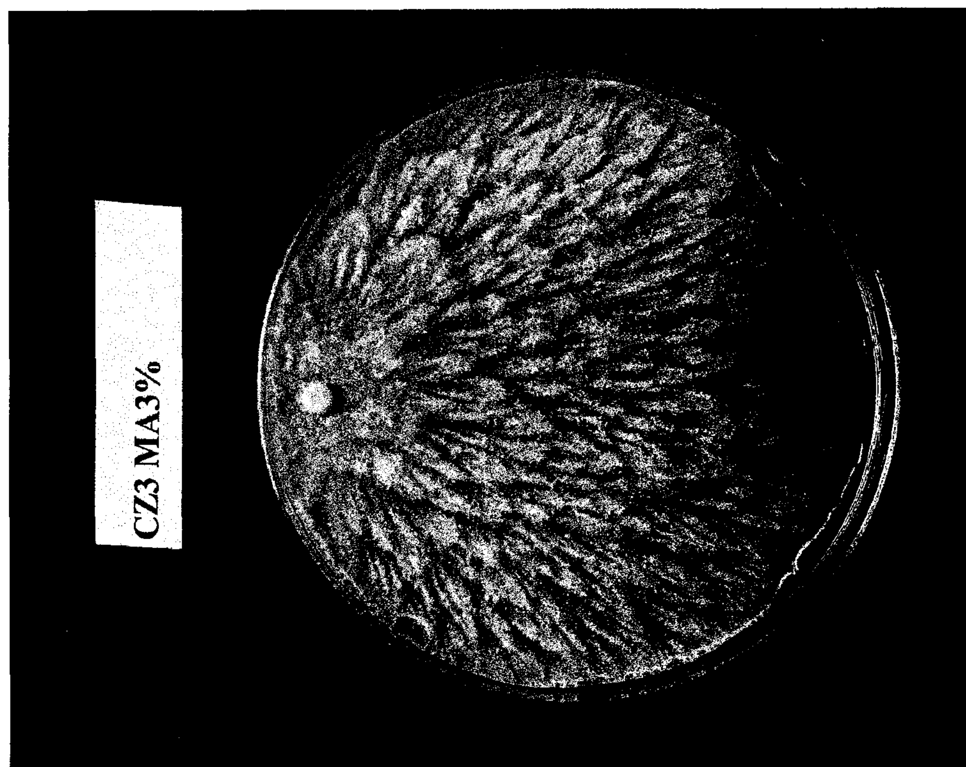

Figure 2.4. Growth of isolate CZ-3 on MA3%.

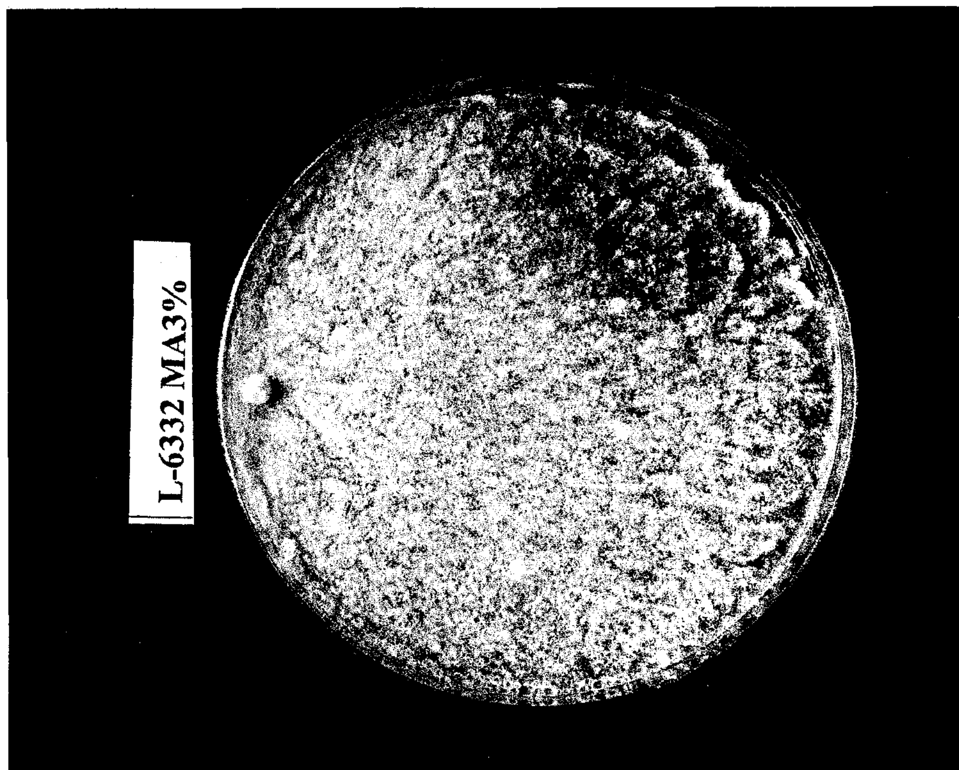

Figure 2.5. Growth of isolate L-6332 on MA3%.

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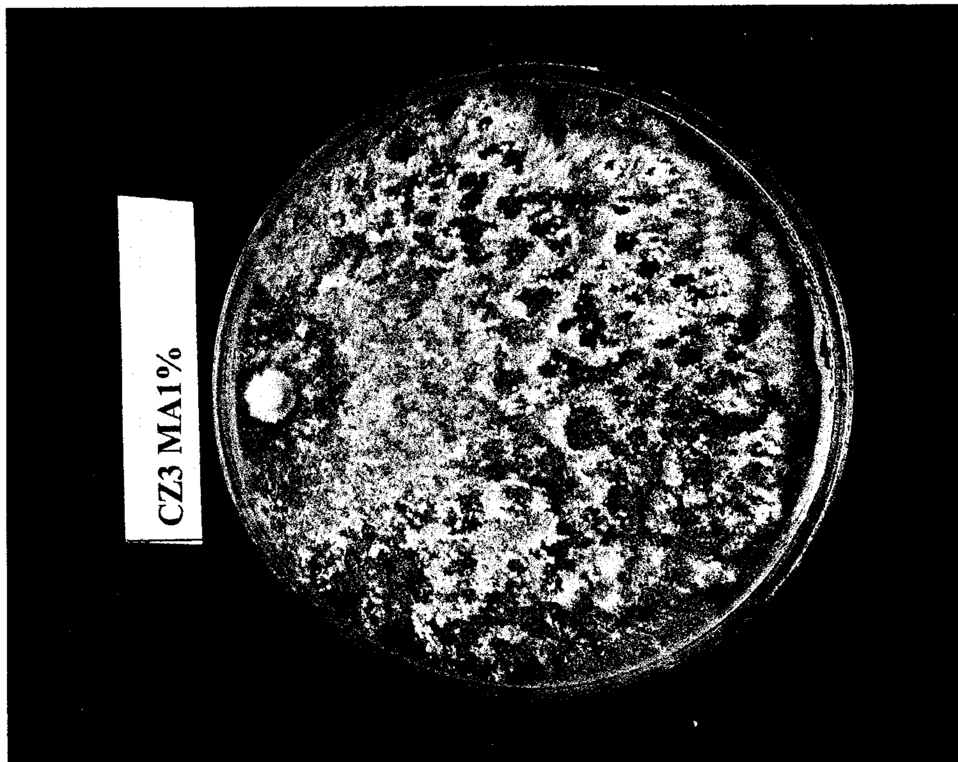

Figure 2.6. Growth of isolate CZ-3 on MA1%.

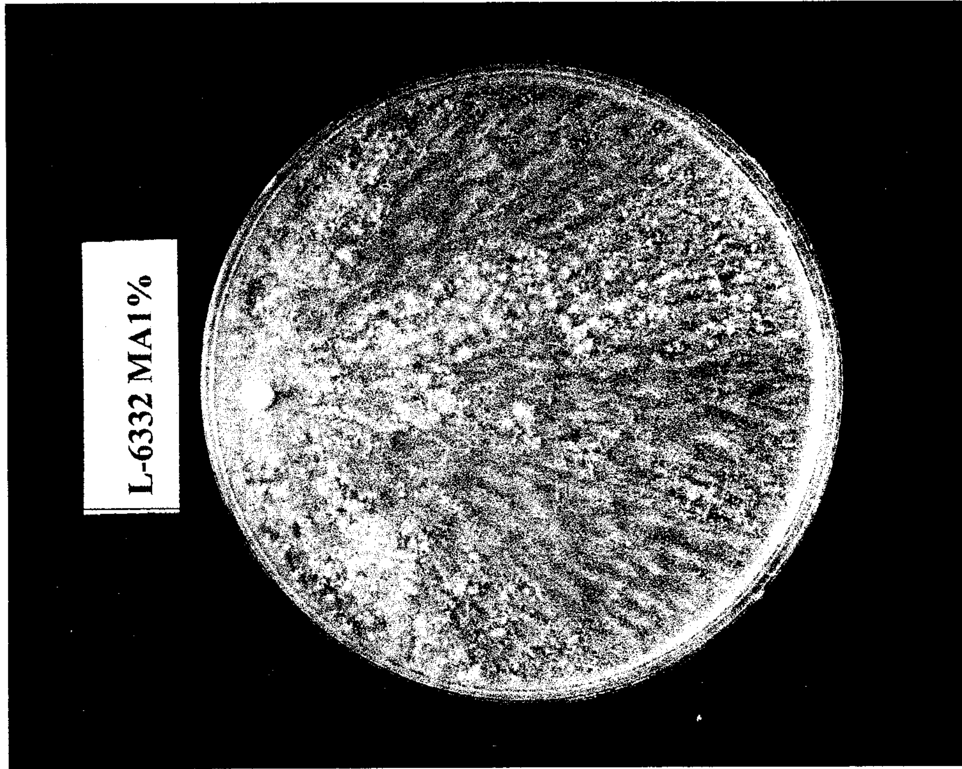

Figure 2.7. Growth of isolate L-6332 on MA1%.

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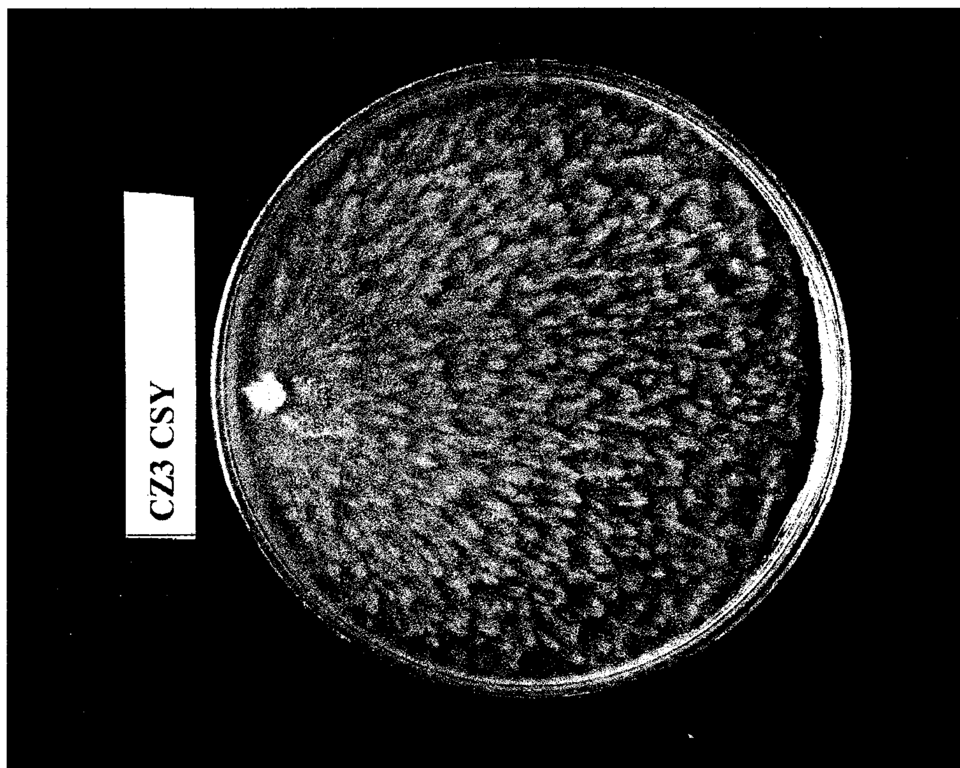

Figure 2.8. Growth of isolate CZ-3 on CSY.

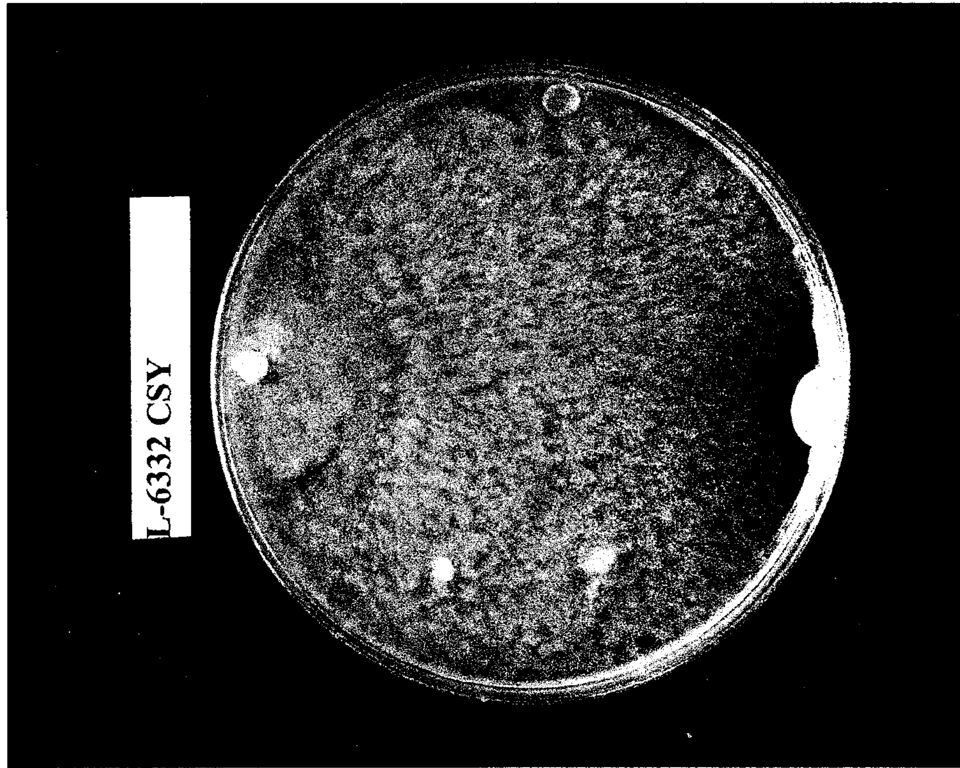

Figure 2.9. Growth of isolate L-6332 on CSY.

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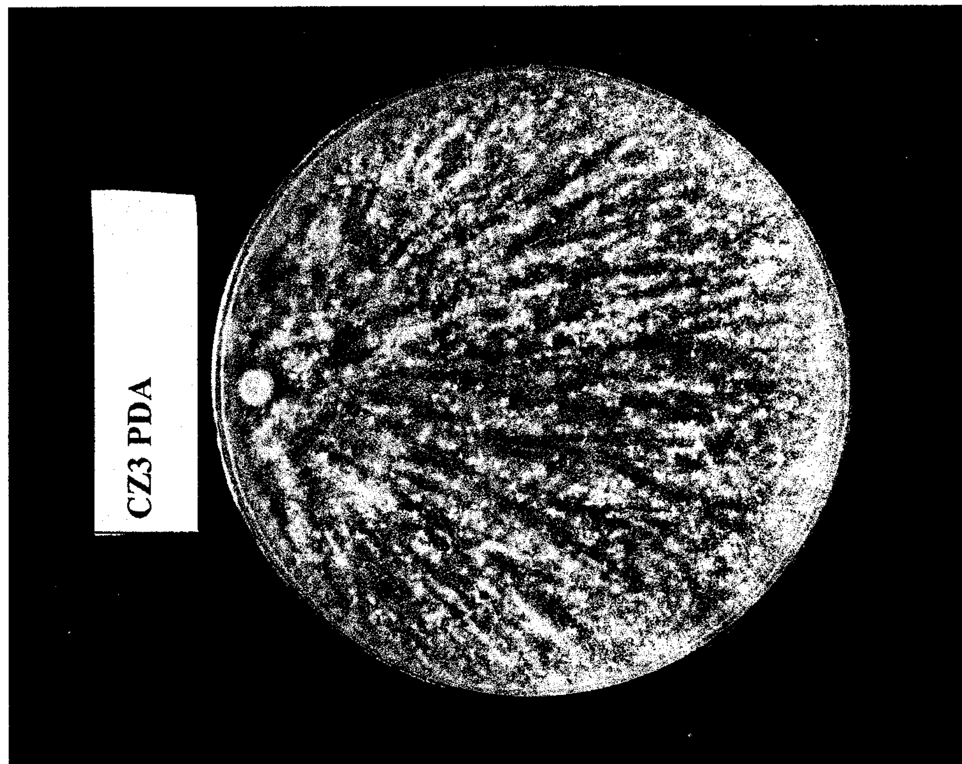

Figure 2.10. Growth of isolate CZ-3 on PDA.

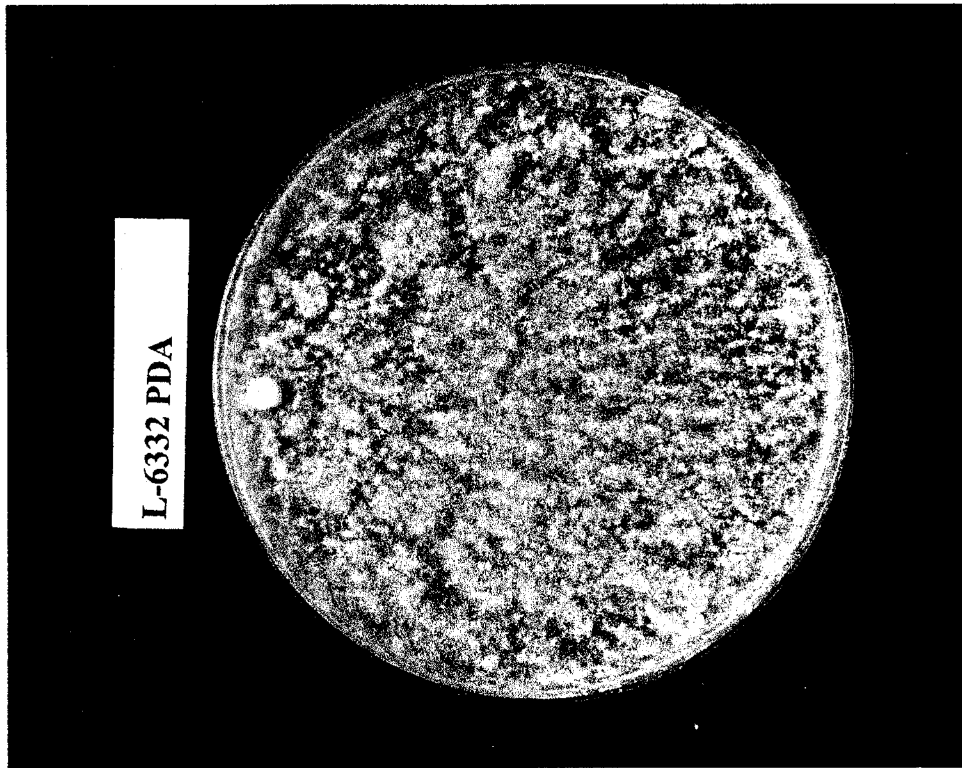

Figure 2.11. Growth of isolate L-6332 on PDA.

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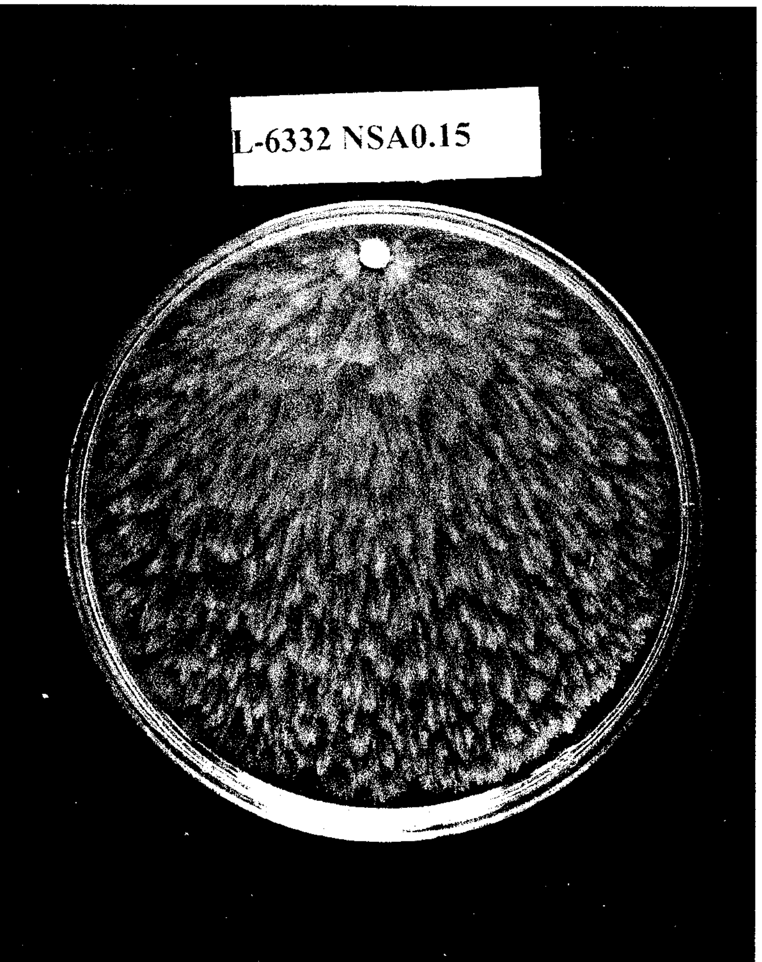

Figure 2.12. Growth of isolate L-6332 on NSA0.15.

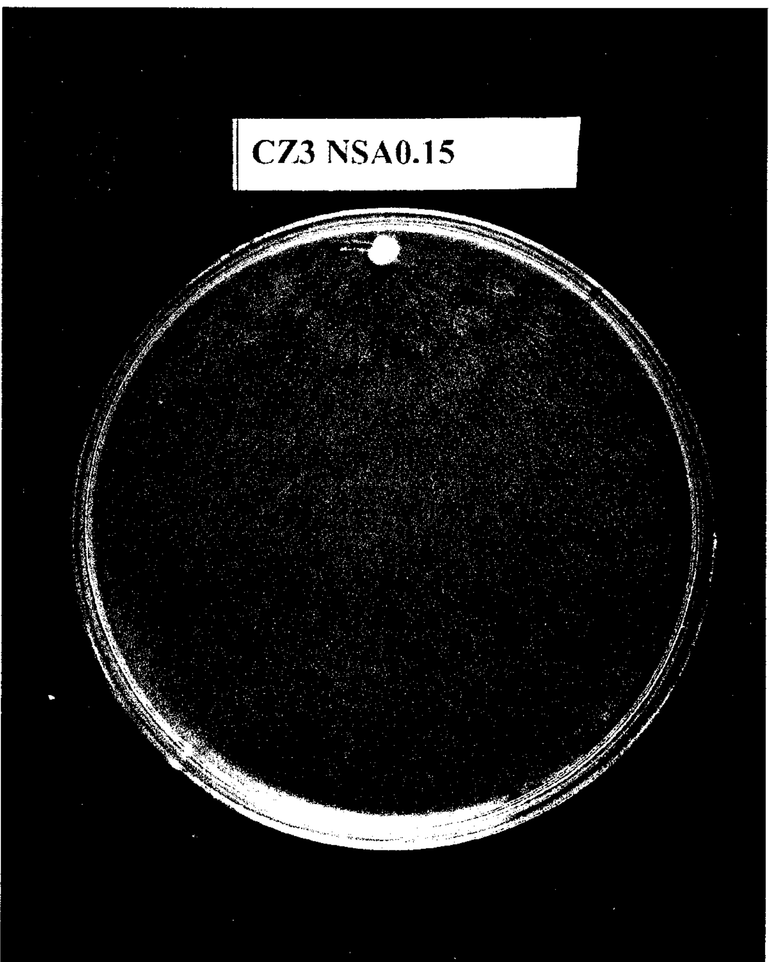

Figure 2.13. Growth of isolate CZ-3 on NSA0.15.

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assembled in long streaks with isolate CZ-3, while L-6332 produced no distinct array of growth (see Figure 2.10 and 2.11). Bleaching of the medium occurred in less than two weeks but a faint yellow tinge remained.

Mycelial mats produced on NSA0.15 were completely appressed with a moist texture and translucent colour. L-6332 generated a fan-like pattern radiating from the point of inoculation consisting of long and short hyphal groupings (see Figure 2.12). No pattern was produced by isolate CZ-3, hyphal groupings were extremely fine and very difficult to identify (see Figure 2.13). The advancing hyphae for both isolates produced an extremely uniform margin of growth. No discolouration of the medium was observed after one month of growth.

Microscopic analysis of cultures

No microscopic differences were observed between isolates CZ-3 and L-6332. Observations of hyphae formed by *C. subvermispora* were categorized into three distinct types of hyphae *viz.*,

a) Primary hyphae: approximately 5-8.5μm wide (Figure 2.14), thin-walled (rarely thick-walled), rarely clamped, regularly simple septate in mature sections irregularly simple septate at the margin of growth and laden with vesicles (especially at hyphal tips) (Figure 2.15);
b) Secondary hyphae: highly branched, located further back from advancing zone, 2-4μm wide, thin walled, and inconsistently clamped at each septum (clamps approximately 4-5μm in diameter) (Figure 2.23). Haploid isolates were observed to be extremely prolific in forming chlamydospores;

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c) Thin/straight hyphae: usually located further back from advancing zone, 1-1.5μm thick, thin walled, rarely branched and regularly clamped at each septum with each clamp a long distance apart (Figure 2.17).

Chlamydospores were both terminal (at hyphal tips (Figure 2.18)) and intercalary (amidst the hyphae (Figure 2.19)), approximately 6-17μm in diameter, occurring sparsely to frequently (Figure 2.20) and mainly spherical in shape sometimes appearing oval or "pear-like" as described by Domanski (1969). The occurrence of chlamydospores was inconsistent on MA1%. For example, isolate CZ-3 was grown on three different plates of MA1% resulting in one plate producing large amounts chlamydospores while the other two plates produced very little. Similar results were observed on PDA and MA3%.

Hyphal swellings were found quite frequently in the secondary hyphae. Some swellings appeared to be newly forming branches while others resembled bumps and made for irregularly shaped hyphae.

Hyphal knots or bulbils (Stalpers 1978; Nobles 1948) were a regular phenomenon that seemed to develop from extensive branching forming twisted clumps of hyphae in distinct areas (Figure 2.21). Similarly, hyphal threads were commonly found as long masses of tightly intertwined hyphae that included a mixture of primary, secondary and thin/straight hyphae (Figure 2.22) and were found in both the advancing and older mycelium. However, both the hyphal knots and threads may be tangles formed during slide preparation for microscopic analysis.

Encrusted hyphae were found regularly consisting of crystals coating the hyphae in large clumps or small individual aggregates (Figure 2.23). Individual crystals were

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found scattered all around the hyphae on the surface as well as within the agar medium. The crystals were shaped as single plate-like structures with a rectangular/trapezoidal appearance (5.5-9.5μm x 2.5-5μm x 1-2μm) (Figure 2.24) and were often fused into clusters of plates that looked like an asterisk viewed from the end (4-9.5μm in diameter) (Figure 2.25). Other commonly observed crystals were octahedral in shape (3.5-10μm in size) (Figure 2.26). In order to confirm that the fungus produced the crystals and not the medium sterile agar plates of media were examined for crystals. Microscopic examination revealed no crystals within the growing media.

The cultural characteristics observed in this chapter were applied to the key code systems of Davidson *et al.* (1942), Nobles (1948, 1965) and Stalpers (1978). Nakasone (1981) developed key codes for *C. subvermispora* using the code systems of Davidson *et al.* (1942) and Nobles (1965). Proposed additions to Nakasone's (1981) codes and Stalpers' (1978) established code are presented in Appendix I.

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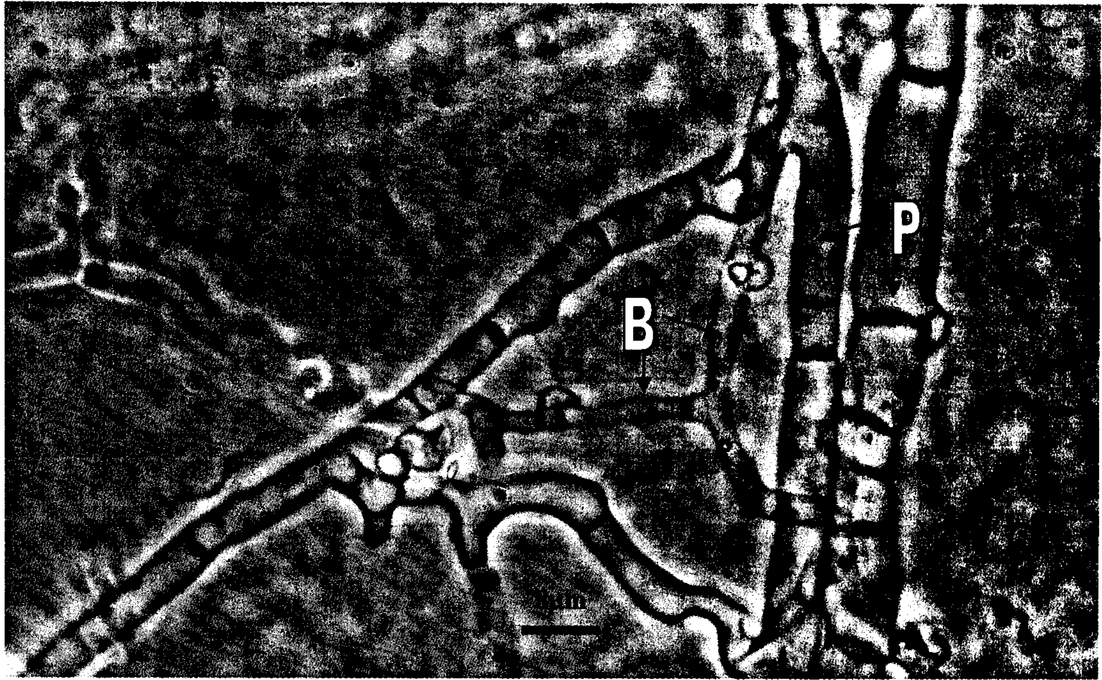

Figure 2.14. Simple septate primary hyphae (P) and clamped secondary branching hyphae (B) of diploid mycelium from *C. subvermispora* grown on 1% malt agar.

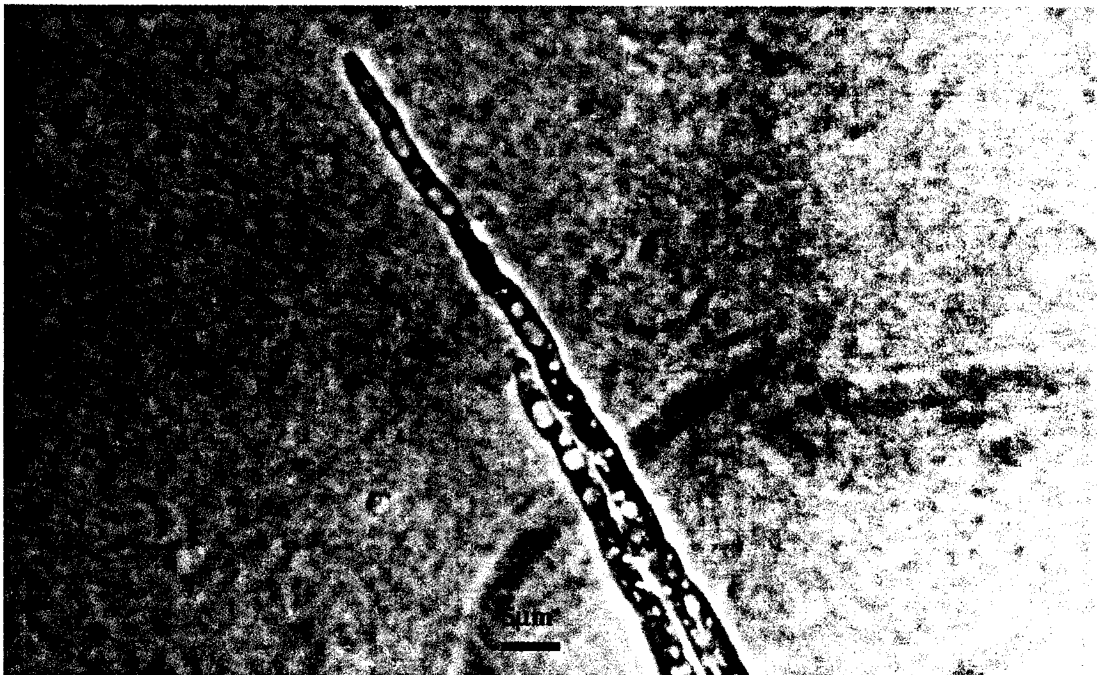

Figure 2.15. Advancing zone hyphae of *C. subvermispora* grown on 1% malt agar. Notice the many vesicles at the hyphal tips.

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Figure 2.16. Diploid secondary hyphae forming clamp connections (C) as well as simple septa (S) from *C. subvermispora* grown on 1% malt agar.

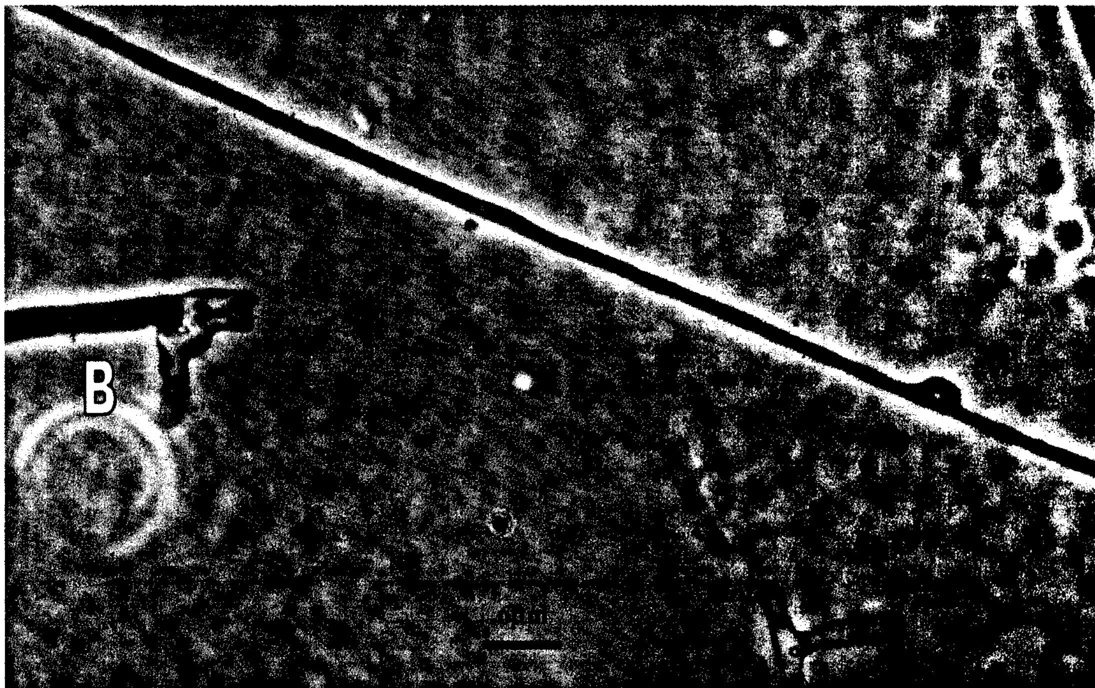

Figure 2.17. Thin/straight hyphae from *C. subvermispora* with a clamp connection. Notice the difference in thickness compared to the secondary hyphae (B). Culture grown on 1% malt agar.

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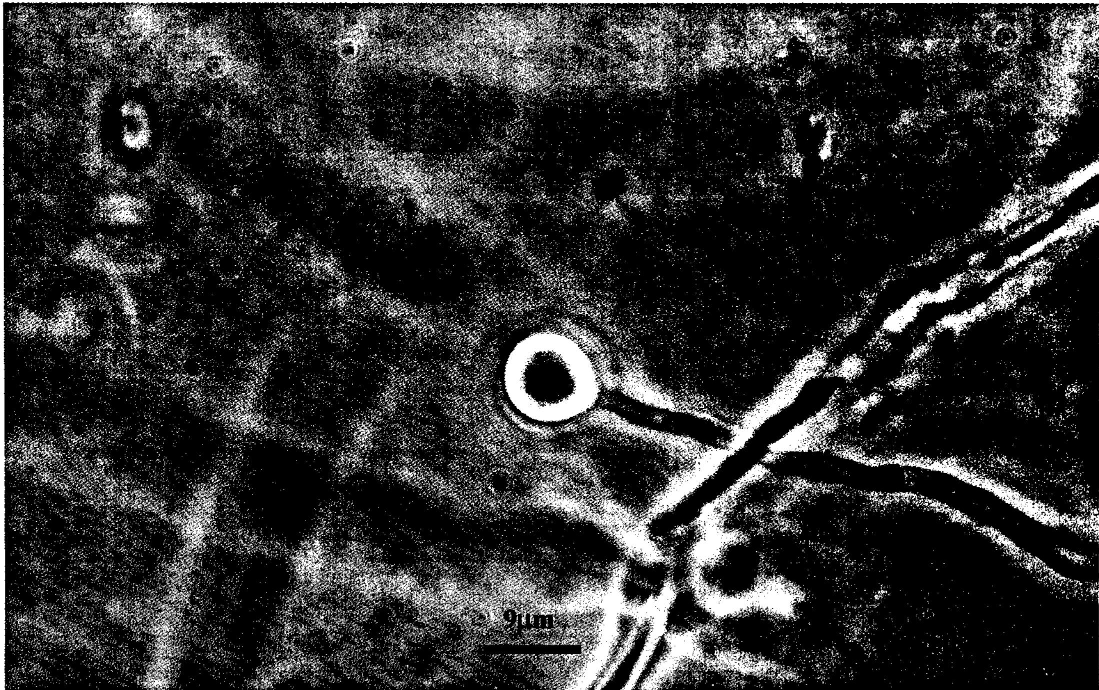

Figure 2.18. Terminal chlamydospore of *C. subvermispora* grown on 1% malt agar.

Figure 2.19. Intercalary chlamydospores of *C. subvermispora* grown on 1% malt agar. Notice the pear-like shape (above) and the spherical shape (below).

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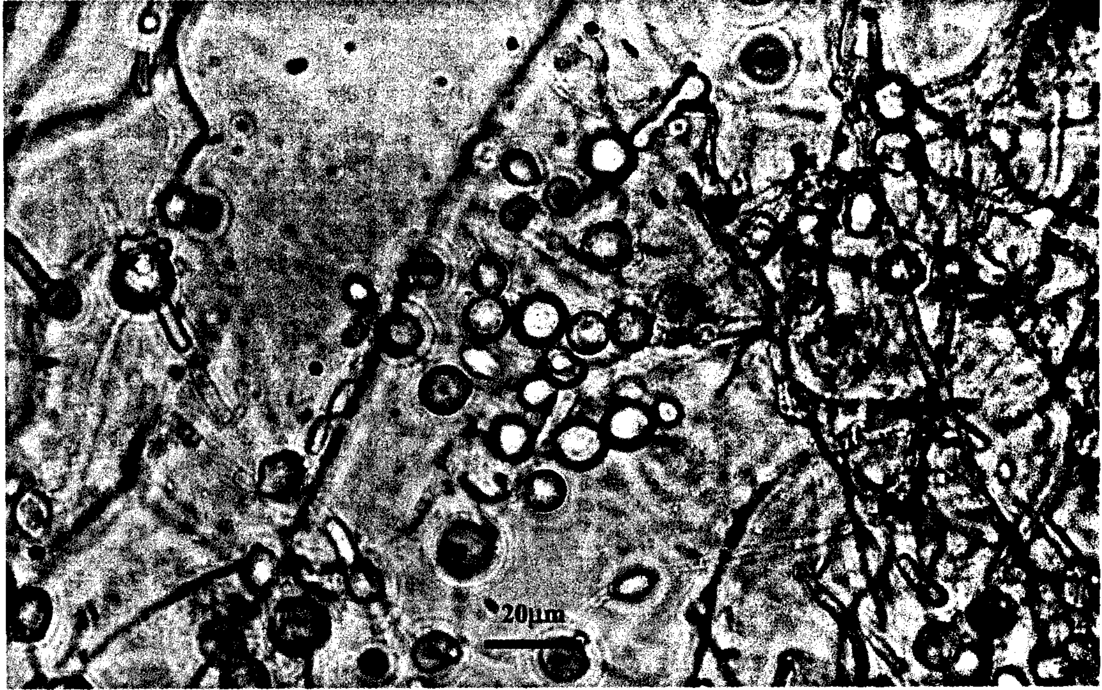

Figure 2.20. High concentrations of chlamydospores from *C. subvermispora* grown on 1% malt agar.

Figure 2.21. Hyphal knot of secondary hyphae from *C. subvermispora* 1% malt agar.

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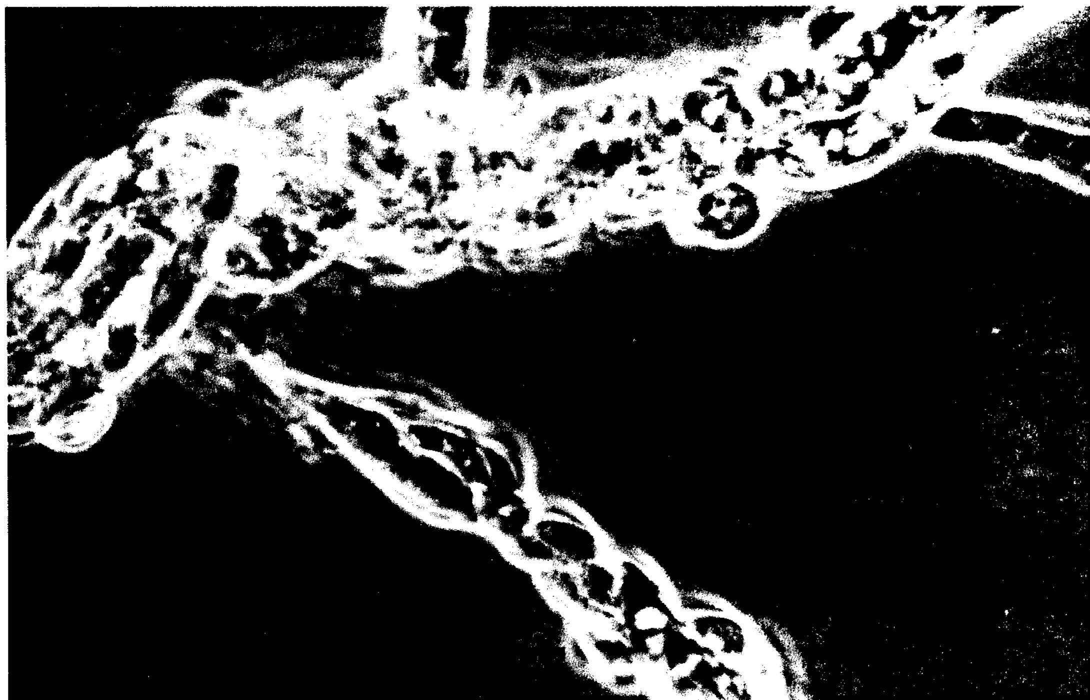

Figure 2.22. Hyphal thread from *C. subvermispora* grown on 1% malt agar.

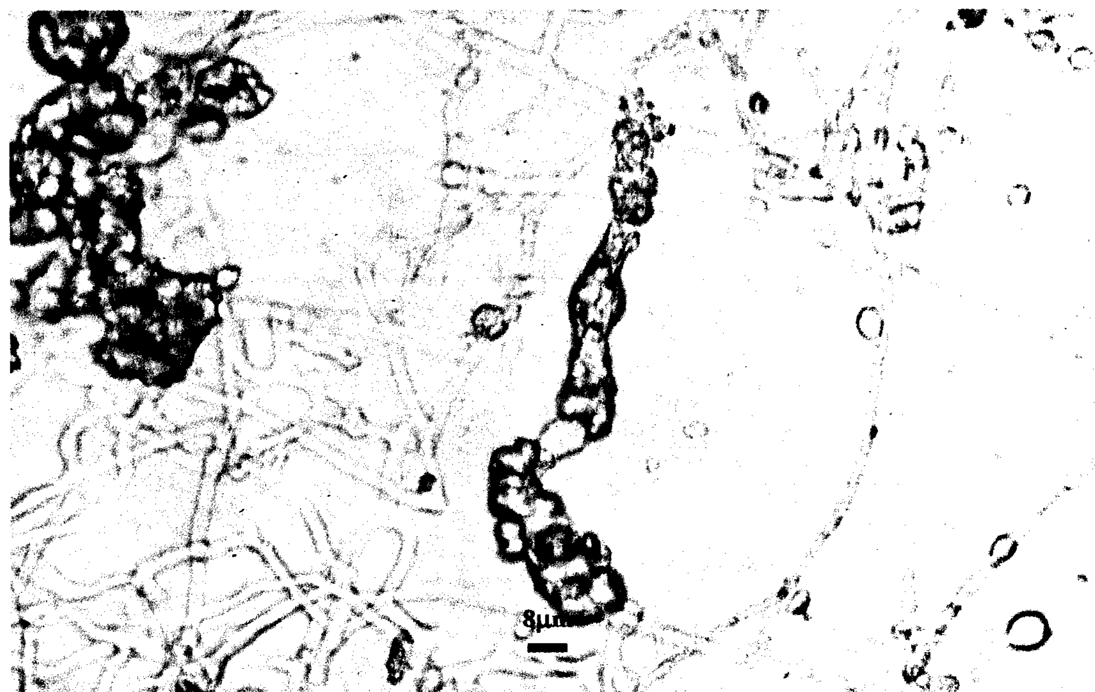

Figure 2.23. Hyphae from *C. subvermispora* encrusted with crystals. Culture grown on 1% malt agar.

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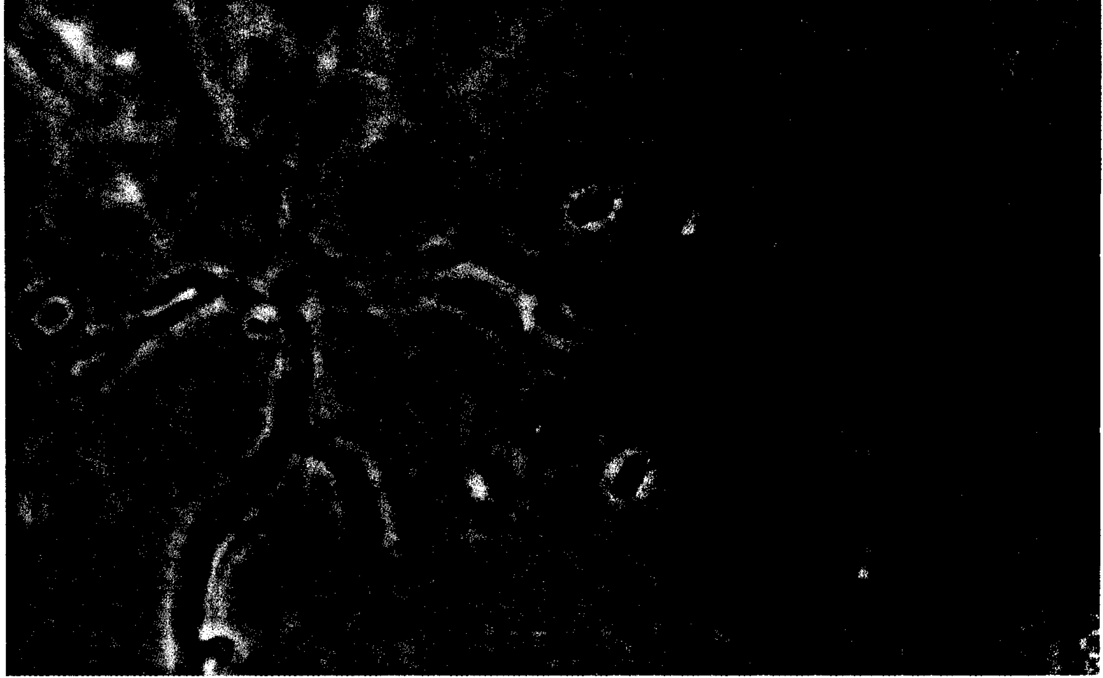

Figure 2.24. Flat plate-like crystals with a trapezoidal appearance produced by the hyphae of *C. subvermispora* grown on 1% malt agar.

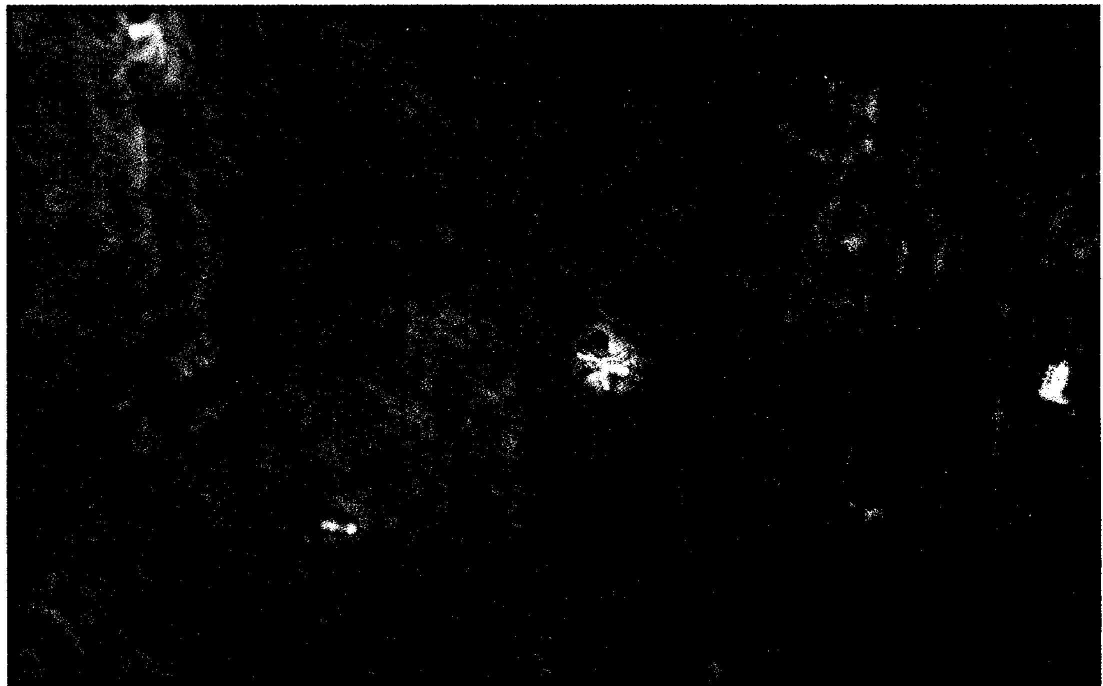

Figure 2.25. Asterisk shaped crystal consisting of flat plate-like crystals formed from the hyphae of *C. subvermispora* grown on 1% malt agar.

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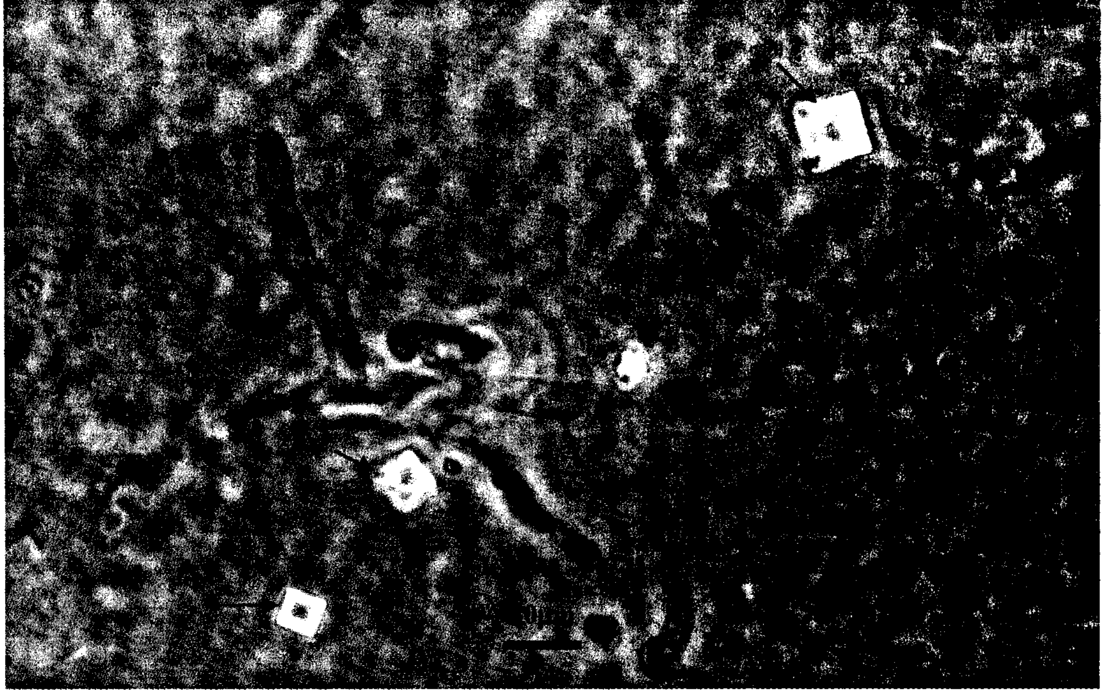

Figure 2.26. Octahedral crystals produced by hyphae from *C. subvermispora* grown on 1% malt agar.

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DISCUSSION

One of the main factors affecting the viability of biopulping is maintaining a constant temperature across the chip piles (Scott *et al.* 1998a). Unventilated chip piles have been found to reach up to 42°C at the center of the pile (Scott *et al.* 1998a). *C. subvermispora* will not survive at temperatures of 40°C or higher. Furthermore, a dramatic decrease in growth occurs between 30 and 35°C. Scott *et al.* (1998a) ventilated chip piles using tubular reactors and was able to stabilize temperatures between 25 and 35°C throughout. Thus, the potential for maintaining chip pile temperature is possible. At lower temperatures *C. subvermispora* does not grow well. At 10°C the fungus is completely inactive and at 15°C growth is severely limited. Such a narrow range of temperatures for the growth of *C. subvermispora* presents problems in biopulping applications in colder climate countries and methods of capturing heat of respiration in chip piles is an important area of research in north temperate climats.

Optimum growth of isolate CZ-3 occurred at 30°C covering an 8.8cm petri dish in 9 days at an approximate rate of 0.98cm/day. The growth rate determined at 25°C for isolate CZ-3 can be compared to the growth rates of other fungi used by Davidson *et al.* (1942) and Wang and Zabel (1990). On a medium of 1.5% malt extract *C. subvermispora* is considered a moderately rapid fungus growing at rate of 8.8cm in 13 days. Furthermore, growth rates at 25°C and 20°C can be used for relative comparisons

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with growth rates used by Nobles (1965) and Stalpers (1978) respectively since the methods herein closely follow their work.

Cultural characteristics of fungi on different media have not been widely studied. Macroscopic observations usually involved the use of only one media for identifying characteristic features. This chapter shows that on different media *C. subvermispora* produced completely different marcroscopic attributes. Media such as MA3% and PDA produced large amounts of aerial hyphae with distinct patterns of growth. Production of aerial hyphae was not as evident with MA1%, while CSY and CS0.15 produced mainly appressed mycelium with occasional small patches of aerial hyphae. No aerial hyphae developed on NSA0.15. Slight differences in mycelial characteristics were observed between the different isolates and this indicates the morphological expression to be under genotypic control. The most distinct comparison was the growth on NSA0.15 and here L-6332 produced a plumose pattern of growth whereas the hyphal growth of CZ-3 was translucent and barely visible.

Cultural characteristics of *C. subvermispora* by Domanski (1969), Stalpers (1978) and Nakasone (1981) were fairly accurate; however, some distinctive characteristics were found further to their work. Detailed observations of this fungus found three distinct hyphal characteristics. Stalpers (1978) and Domanski (1969) did not observe thin/straight hyphae. Both authors did comment on the highly branched nature of the hyphae and Domanski did find the rarely occurring thick walled hyphae. Nakasone (1981) distinguished between the hyphae submerged within the medium and the aerial hyphae. Nakasone (1981) categorized the aerial hyphae as encrusted, very thin binding hyphae or rare thick-walled fiber hyphae. The very thin binding hyphae may be similar to the thin/straight hyphae; however, Nakasone observed the binding hyphae to

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be simple septate and highly branched. The thin/straight hyphae found with isolates CZ-3 and L-6332 were clamped and rarely branched. Domanski (1969) established that hyphae in the advancing zone was rarely septate and consisted of wider hyphae concurring with the results found in this paper. The only difference observed between haploid and diploid mycelium was the vesicular branching hyphae confirming Domanski's observations.

Domanski (1969), Stalpers (1978) and Nakasone (1981) described chlamydospores and encrusted hyphae. Stalpers (1978) found crystaline structures prominently throughout cultures of *C. subvermispora*; surprisingly, Stalpers (1978) did not describe the two distinct crystals commonly found is this study. Likewise, Kirk *et al.* (1993) found large amounts of crystals produced by hyphae of *C. subvermispora* when grown on wood chips which were identified as calcium oxalate. In addition to calcium oxalate, Akhtar *et al.* (1998) discovered occasional accumulations of manganese. Glyoxates and oxalates isolated from *C. subvermispora* were found to be important in lignin degradation (Galkin *et al.* 1998; Urzua *et al.* 1998). Thus, the crystals observed could be any one of these deposits. There is a definite morphological distinction between the plate-like and octagonal crystals, and this suggests at least two different kinds of chemicals.

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CHAPTER 3

DEVELOPMENT OF BASIDIOCARPS AND BASIDIOSPORE PRODUCTION

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INTRODUCTION

In-vitro (in culture) production of fruiting bodies from white-rot basidiomycetes is rather uncommon for most basidiomycetes studied in culture (Croan 1994). *C. subvermispora* is considered by many to be non-fruiting under cultural conditions (Kirk *et al.* 1993; Akhtar *et al.* 1997; Tello *et al.* 2001), and so an effort was made to develop basidiocarps when rudimentary fruiting bodies were found at the edge of a petri dish containing isolate L-6332. Basidiospore production may have significant applications for biotechnology by greatly optimizing the commercialization of the biopulping process. The importance of *C. subvermispora* as a biopulping fungus has led to research in producing large-scale inoculation techniques for pulp chips (Akhtar *et al.* 1997; Jones 1998; Saxena *et al.* 2001). Spores from the Ascomycete *Ophiostoma piliferum* have been commercialized as the product Cartapip™ (Trademark 74033355, 1991) to reduce pitch and resin in pulp chips prior to mechanical pulping (Farrell *et al.* 1992; Blanchette *et al.* 1992; Fischer *et al.* 1994). The success in biopulping is greatly improved by having massive numbers of spores that can be used for inoculating wood chips on a commercial scale.

There are many techniques that have been used to induce basidiocarp formation. Croan (1994) found that carbon and nitrogen limitation induced fruiting body formation in the white-rot fungi *Schizophyllum commune*, while *Trametes versicolor* was only affected by nitrogen limitation. Air and light exposure also seemed to have a definite

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effect on prompting basidiocarp formation in both fungi. Rayner and Boddy (1988) identified aeration, exposure to light, type of substrate and temperature as the four main external factors that influence fruiting. Wessels (1994) found exposure to light had variable effects on different fungi, though high oxygen levels consistently produced positive results.

As well, there are many benefits that can be derived from fruiting bodies in the laboratory for basic research. For this study it was imperative to find some means to induce basidiocarp development in order to produce basidiospores for incompatibility or sexuality testing (see Chapter 4). Understanding the nuclear life history of the fungus is another avenue of research but was not undertaken extensively in this study.

This chapter describes the methods, materials and results for five studies that were undertaken with regards to basidiocarp formation and basidiospore production of *C. subvermispora*. The five studies are: fruiting by individual isolates, fruiting by agar media, fruiting on *Pinus banksiana* boards, spore production by isolate and spore germination by isolate. A discussion of the implications of these results follows.

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METHODS

Fruiting by individual isolates – tray method

Four 1%MA plates (petri dishes) were aseptically inoculated with each of the four isolates: CZ-3, L-6332, FP-90031-sp and ME-485. The plates were placed in clear plastic bags for moisture retention and incubated at 27°C. After the plates had completely grown over with mycelium, they were transferred to an aeration rack at room temperature (ca. 25°C). The aeration tray consisted of a tray covered in paper towels with glass rods placed upon the towels. The towels and glass rods were sterilized in an autoclave for 15 minutes at 120°C and 20 psi, while the plastic trays were rubbed down with 1% a.i. sodium hypochlorite to reduce contaminants. Prior to adding the petri dishes, sterile water was applied to the towels until they were completely moist. This was done to maintain high humidity so as to prevent desiccation of the dishes.

Two aeration trays were constructed to hold all 20 petri dishes. The racks were able to hold a dozen 8.8 x 1.5 cm plastic petri dishes when arranged four across and three down. Each dish had the cover removed and was placed upside down on two glass rods that extended the length of the tray. The glass rods were used to keep the dishes elevated away from the moist towels in order to provide adequate aeration and avoid contamination from direct contact with the paper towels. Each dish was randomly placed on either of the two trays to alleviate any bias or unknown limiting factors between the two trays. Once in place, the rack was covered with transparent plastic to reduce contamination.

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The racks were examined twice daily for fruiting and checked for moisture. Sterile water was added to the towels as needed. This also allowed for re-aeration of the racks before covering them again. Exposure to light occurred on a normal 24-hour daylight period during the winter months.

Fruiting by agar media – tray method

The two isolates, L-6332 and CZ-3, that produced successful fruiting in the previous experiment were examined for the effect of media on fruiting. The six media mentioned in Chapter 2 (MA1%, MA3%, PDA, CS0.15, CSY and NSA0.15) were used for this experiment. Three replicates were established for each medium and each isolate. This experiment followed the same procedure as described above. All plates were randomly placed on the aeration rack at room temperature (ca. 25°C) and were observed.

Over the course of two months, each plate was examined daily for the onset of fruiting and abundance of fruiting bodies. Due to this daily exposure, contamination became an increasing problem as the study proceeded. Petri dishes were numbered to avoid confusion and contaminated plates were replaced with fresh plates.

Fruiting on *Pinus banksiana* boards

Two 2x6 jack pine boards each were cut into three 24-inch lengths. All six pieces were soaked in a 0.5% glucose and 1% yeast extract broth for 30 minutes. Paper towels soaked in the same broth were wrapped around each board to help retain the moisture in the boards during sterilization. The boards were then divided into two autoclavable bags. The bags were sealed and autoclaved as usual. After sterilization, the boards were taken out of the autoclave and allowed to cool. They then were unwrapped in a laminar flow hood and some of the paper toweling was aseptically

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pealed back from the boards. Plugs of CZ-3 mycelium on 1% malt agar were placed on four different spots on each board. The boards were then carefully resealed inside the autoclavable bags and left to sit at room temperature (ca. 25°C) in the teaching laboratory. Once the fungus was well established on the boards, they were removed from the autoclavable bags and transferred to an incubator.

To induce fruiting, the exposed boards (paper towel wrapping removed) were placed in an incubator that was wiped down with a 10% bleach solution to reduce any contaminants. In the incubator, each of four racks was surrounded with transparent plastic so that a greenhouse-like chamber was created. Blocks were placed at both ends of each board so that both top and bottom sides were exposed to the air. Another layer of sterile paper towels was added to the upper surface of the board and soaked with sterile water to elevate humidity within the incubator and prevent drying out of the board. On the bottom of each chamber was a layer of moist sterile paper to increase the moisture content. Folding and overlapping the plastic lightly sealed the entrance of each chamber. The incubator was set at 27°C (see Figure 3.1). The boards were checked daily for fruiting and watered with a misting bottle containing sterile water. Exposure to light and fresh aeration only occurred during these daily checks.

Once fruiting bodies began to form on the boards, thin sheets of plastic were placed underneath the basidiocarps on a daily basis to collect spore prints.

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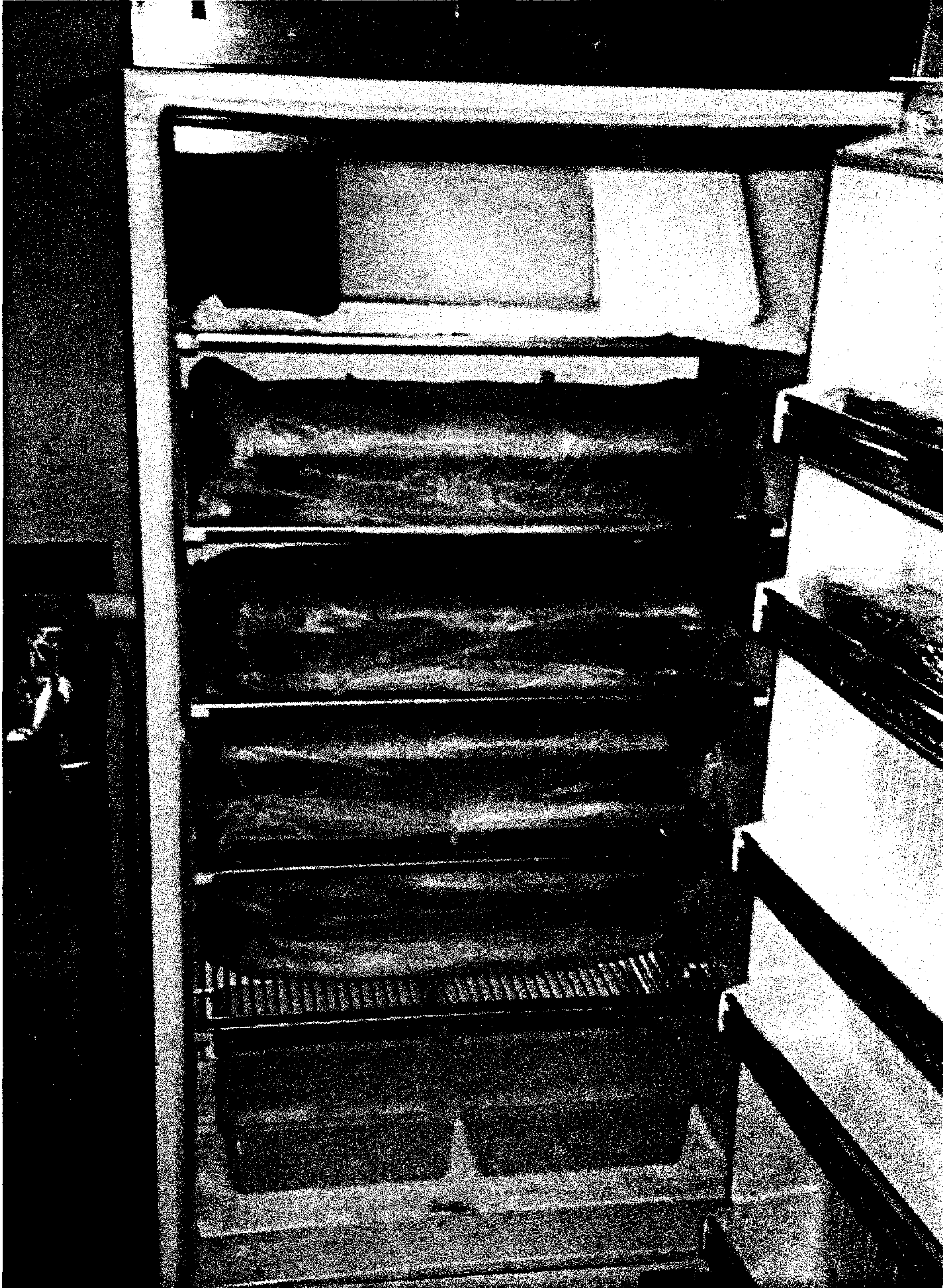

Figure 3.1. Incubator with four chambers containing jack pine boards inoculated with *C. subvermispora*. The top two chambers contain two boards while the bottom two contain one each. Notice the dark coloured support blocks at each end of the boards and the layer of paper towel on top of the boards and covering the bottom of the chamber. In this case the chambers are closed with the plastic folded over keeping the moisture inside.

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Spore Production by Isolate

Basidiocarps of L-6332 and CZ-3 developed on 1% malt agar plates after 3-4 weeks and were used as the source of basidiospores for later studies of sexuality. Also attempts were made to establish and evaluate basidiospore production. Plugs of agar of two different diameters (0.6cm and 0.8cm) containing basidiocarps of relatively similar thickness were attached to the underside of a petri dish lid and placed over a cover slip for 24-hours. Spores accumulated during this period were thoroughly rinsed off the cover slips in 20mL of sterile water. To measure spore production an AO Spencer® Bright-Line Hemacytometer was used as follows:

$$\frac{\text{\# of spores counted on grid x 4000}}{80} = \text{spores/cc}$$

spores/cc x 20mL of water = total spore count over 24 hours

where: 4000 is a constant and 80 represents spores counted in 80 blocks on the grid

The procedure was repeated twice for each plug size in order to reduce error.

Spore Germination

Small sections of basidiocarps that developed on 1% malt agar were removed and attached to the underside of a petri dish lid with Vaseline®. Basidiocarps of CZ-3 and L-6332 were placed over plates of 1% malt agar. The petri dishes were placed in an incubator at 27°C and after a 24-hour period the agar plates were examined under a dissecting microscope for spore prints. Three different spore prints were examined for each isolate. A small squared area of each spore print was delineated on the agar surface with a fine-tipped dissecting needle. Inside this area spores were counted and characterized as swollen and/or ungerminated. The same areas were examined again

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after 48-hours. Spores where counted and characterized as: swollen, germ tube formation, advanced germination (germination with lateral branching) and non-germinating. Spores with germ tubes or more advanced germination were used to estimate percent germination.

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RESULTS

Fruiting by individual isolates – tray method

Basidiocarps were first observed for isolates L-6332 and CZ-3 after 4 weeks in the aeration racks. A week later fruiting was observed in all four replicates for these two isolates (see Table 3.1). Isolates FP-90031-sp and ME-485 showed no signs of fruiting after weeks 4 and 5. Both isolates were kept in the chambers for another 4 weeks and produced no results.

Table 3.1. Fruiting by isolate over 4-and 5-week periods.

Isolate	Petri Dish	4 weeks	5 weeks
L-6332	1	Fruiting	Fruiting
	2	Fruiting	Fruiting
	3	-	Fruiting
	4	-	Fruiting
CZ-3	1	Fruiting	Fruiting
	2	-	Fruiting
	3	Fruiting	Fruiting
	4	Fruiting	Fruiting
FP-90031-sp	1	-	-
	2	-	-
	3	-	-
	4	-	-
ME-485	1	-	-
	2	-	-
	3	-	-
	4	-	-

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Fruiting for isolates L-6332 and CZ-3 started in isolated areas forming pores on the medium from hyphal tufts or sporadically in areas with no aerial hyphae. Some basidiocarps were isolated to small sections on the medium surface with no development elsewhere in the dish. Other situations showed the majority of the medium being cover with a thin poroid basidiocarp layer and/or single raised tufts of basidiocarps scattered on the agar.

Fruiting by agar media – tray method

Basidiocarp formation occurred with three of the six media. Consistency was the factor separating the three successful media, 1% malt agar (MA1%) produced fruiting bodies with all three replicates for both isolates and was by far the most consistent medium (see Table 3.2). Potato dextrose agar (PDA) and 3% malt extract agar (MA3%) formed fruiting bodies in only one replicate per isolate.

After a week of aeration the aerial hyphae seemed to increase with MA1%, MA3% and PDA. The other media did not distinctly produce aerial hyphae. Aerial mycelium produced cottony white patches or tufts on the 1% and 3% MA, and it was from these patches where basidiocarp formation took place and developed. The PDA produced aerial hyphae which was mostly restricted to tufts raised above the agar surface. From these tufts small basidiocarps developed.

The rapidity of fruiting body formation was difficult to predict. Isolate CZ-3 produced basidiocarps just after 2 weeks for both MA1% and MA3% while the PDA lagged for almost a month. For L-6332, only one plate formed a fruiting body on MA1% after 2 weeks, while the other two plates delayed for 28 days. Although only one plate of MA3% formed a basidiocarp, surprisingly it took the least amount of time

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Table 3.2 Fruiting observations and abundance for six media and two isolates of *C. subvermispora*.

Isolate	Medium	Replicate	Basidiocarp Formation	Abundance
CZ-3	MA1%	1	+ 15 days	good
		2	+ 15 days	good
		3	+ 15 days	good
	MA3%	1	-	slight
		2	+ 15 days	
		3	-	slight
	PDA	1	-	
		2	-	
		3	+ 27 days	slight
	NSA0.15	1	-	
		2	-	
		3	-	
	CSY	1	-	
		2	-	
		3	-	
	CS0.15	1	-	
		2	-	
		3	-	
L-6332	MA1%	1	+ 28 days	good
		2	+ 28 days	good
		3	+ 15 days	good
	MA3%	1	-	
		2	-	
		3	+ 12 days	good
	PDA	1	+ 28 days	slight
		2	-	
		3	-	
	NSA0.15	1	-	
		2	-	
		3	-	
	CSY	1	-	
		2	-	
		3	-	
	CS0.15	1	-	
		2	-	
		3	-	

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(e.g. 2 weeks). Fruiting was successful on a single plate of PDA and was consistent with isolate CZ-3 lingering for 28 days.

Basidiocarp abundance was based on the area of agar surface covered. The 1% malt agar plates provided the highest abundance of fruiting for both isolates by covering half to three quarters of the agar surface (see Figure 3.2 and 3.3). Minimal fruiting was observed with isolate CZ-3 on 3% malt agar and both isolates on PDA. Basidiocarp formation was very patchy limited to two or three single tufts of aerial hyphae (see Figure 3.4). Unexpectedly, isolate L-6332 on 3% malt agar formed extensive fruiting bodies covering three quarters of the agar surface.

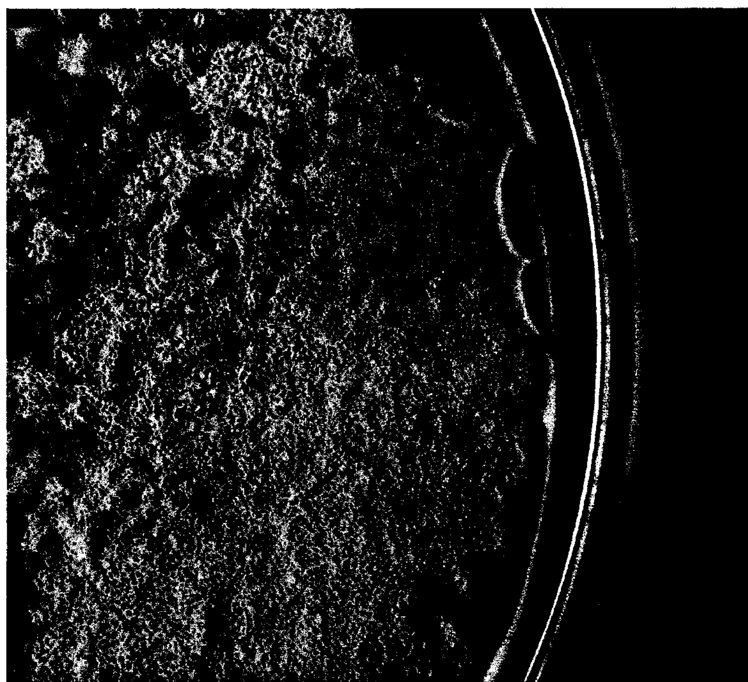

Figure 3.2. Poroid basidiocarp of *C. subvermispora,* isolate CZ-3 on MA1%.

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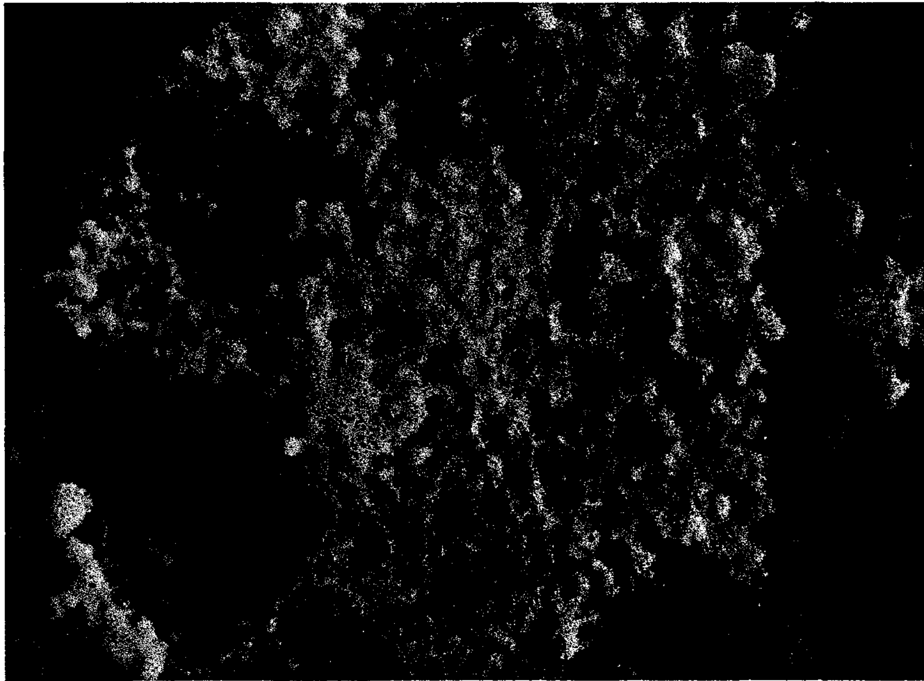

Figure 3.3. Poroid basidiocarp of *C. subvermispora,* isolate L-6332 on MA1%.

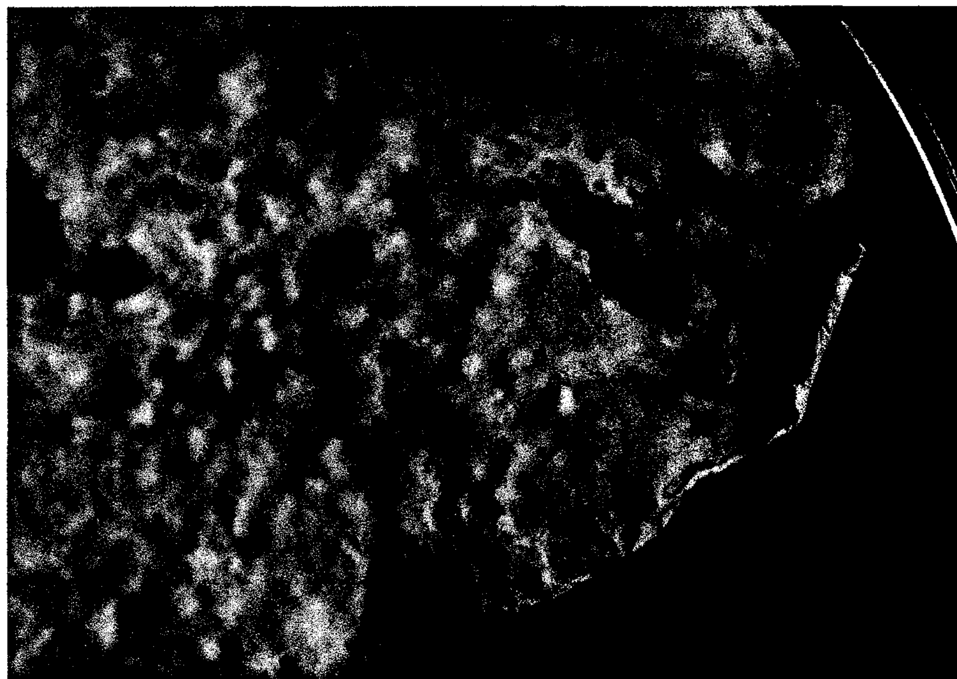

Figure 3.4. Poroid Basidiocarp of *C. subvermispora,* isolate L-6332 on PDA.

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Fruiting on *Pinus banksiana* boards

After 5 months of incubation in the bags at room temperature the boards were extensively covered with mycelial fans and cords (Figure 3.5). Mycelial mats were observed as being felty and smooth in texture as well as white in colour (Figure 3.6).

Fruiting bodies began developing on the jack pine boards after one week of incubation in the growth chambers. Fruiting began on the vertical sides of the boards before forming on the bottom of the boards. Basidiospores were produced in large quantities on a daily basis from the many basidiocarps on the boards.

Basidiocarp formation continued on all six boards for three months. Eventually the boards were colonized by molds and the experiment was terminated. Two boards dried-out and so two basidiocarps became dry, rigid and brittle. When fresh, the fruiting bodies were delicate and soft and easily damaged with the touch of a finger. Basidiocarps were approximately 3mm thick and white to pale cream when fresh. They later dried to a pale yellow colour (see Figures 3.7).

The thin sheets of plastic that were placed underneath the basidiocarps were found to be effective at collecting spore prints on a daily basis (see Figure 3.8).

An unexpected result after removal of the boards from the growth chamber was the occurrence of a fine white powder that covered everything in the incubator chamber. The fine powder was examined under microscope and found to be composed of basidiospores. Figure 3.9 shows a white dusting of spore aggregations that accumulated on a green plastic lid in the incubator over the course of 3 months.

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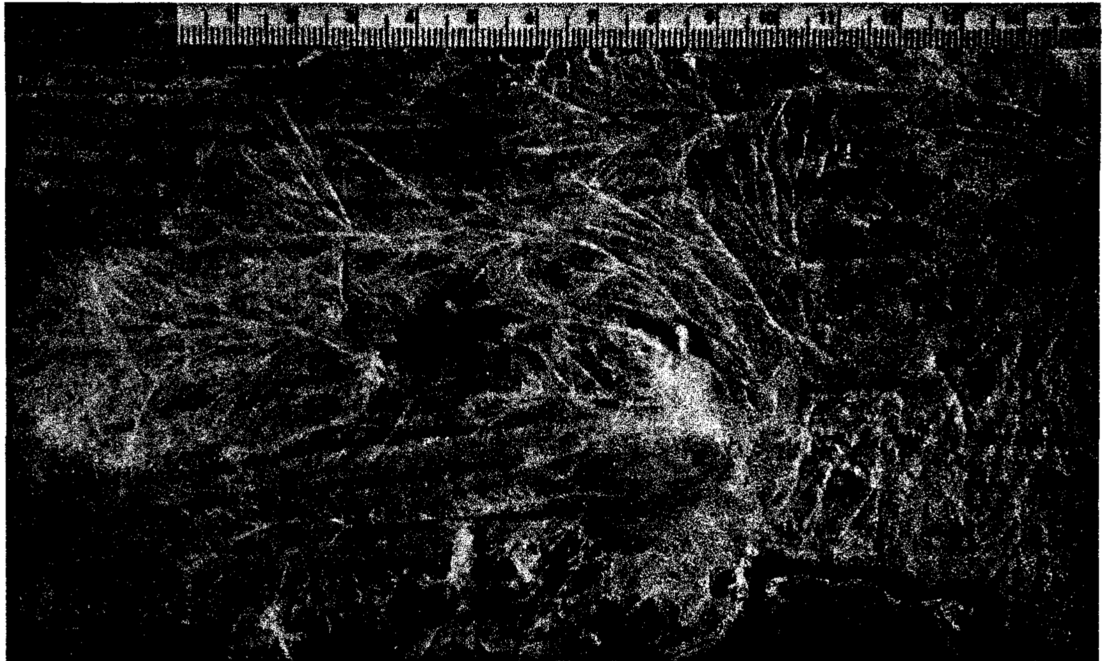

Figure 3.5. Mycelial mat of *C. subvermispora* on jack pine showing fan-like pattern and hyphal cords.

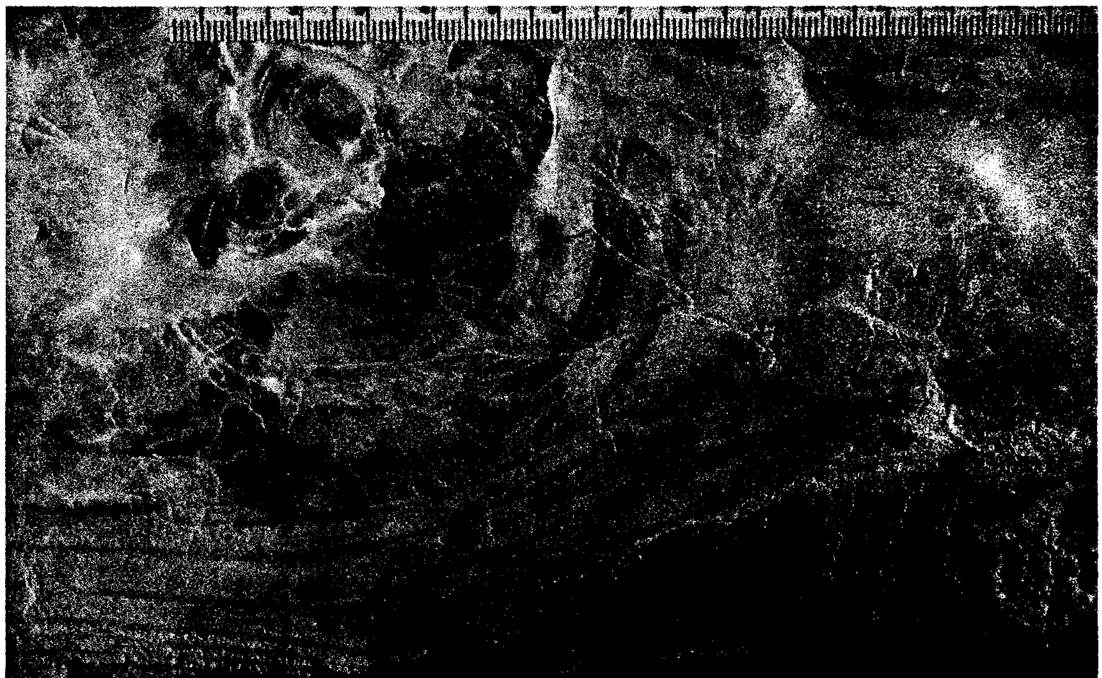

Figure 3.6. Mycelial mat of *C. subvermispora* on jack pine showing white felty texture.

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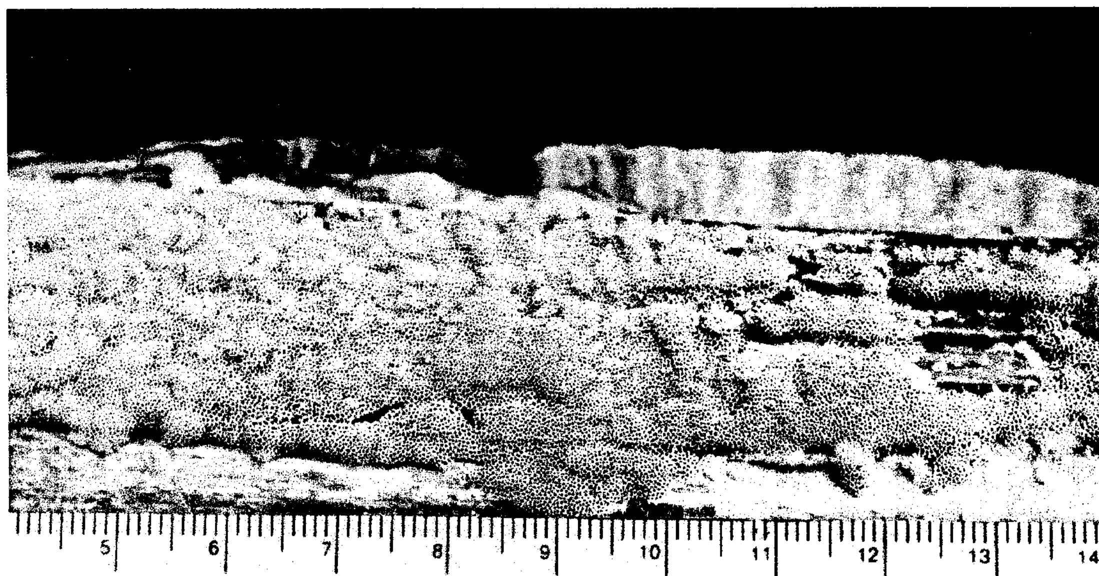

Figure 3.7. Young basidiocarp of *C. subvermispora* approximately 6-weeks-old on jack pine.

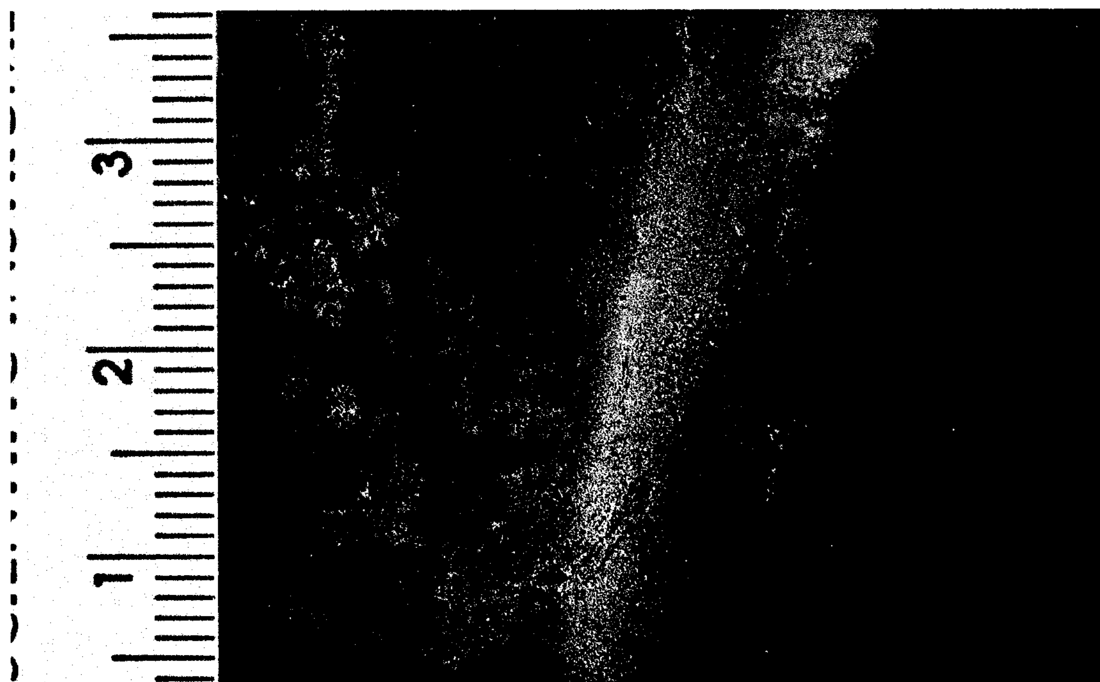

Figure 3.8. Spore print of *C. subvermispora* isolate CZ-3.

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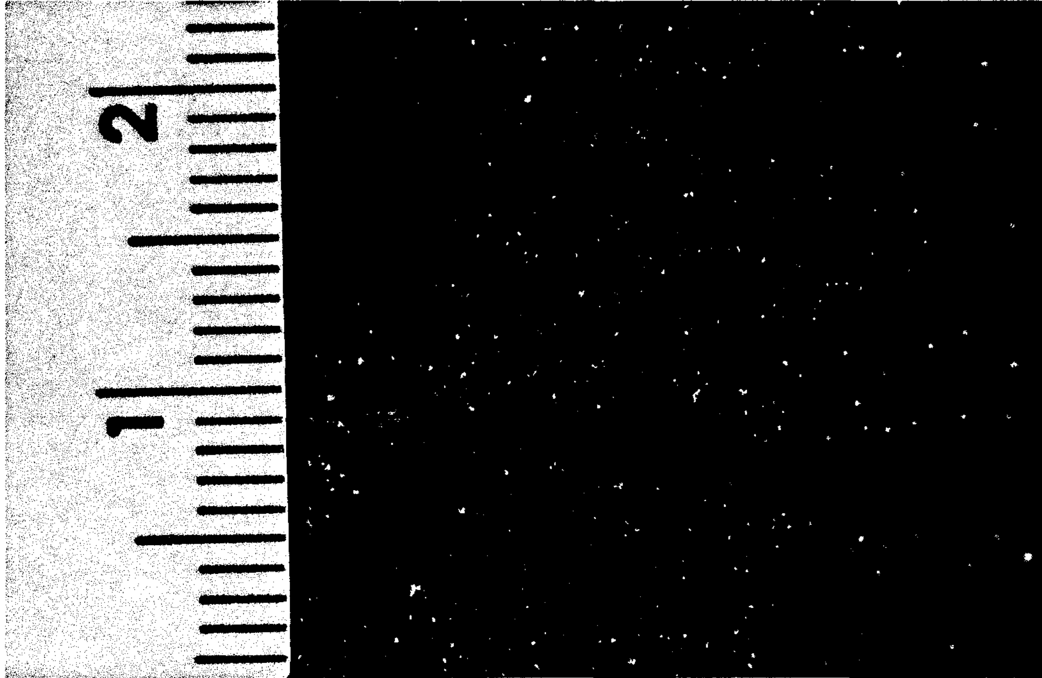

Figure 3.9. Dusting of basidiospore aggregates from isolate CZ-3 found in the incubator.

Spore production by isolate

The 24-hour spore count for both isolates differed greatly. Table 3.3 lists the hemacytometer results for both isolates using the two different sizes of basidiocarp plugs. CZ-3 produced an extremely high concentration for both the small and large diameter plugs ranging between 85,000 – 1,625,000 spores in 20mL of water. L-6332 had much lower results and produced 4,000 – 17,000 spores in the 20mL suspension.

Spore germination

Basidospores from fresh basidiocarps of isolates CZ-3 and L-6332, inoculated at 27°C each, were examined after 24-and 48-hours. The 24-hour period results are listed in Table 3.4. Swollen spores were observed but not classified as germinating spores.

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Table 3.3. Hemacytometer calculations for spore production after 24-hours by isolate using two sizes of basidiocarp plugs.

Isolate	Diameter of plug (cm)	Replicate	Spores counted on hemacytometer	Number of spore in 20mL of suspension
CZ-3	0.6	1	85	85,000
		2	166	166,000
	0.8	1	1116	1,116,000
		2	1625	1,625,000
L-6332	0.6	1	8	8,000
		2	4	4,000
	0.8	1	17	17,000
		2	15	15,000

Thus, germination was calculated at the 48-hour period. After 24-hours, only 10% of the spores from L-6332 were swollen; thereby indicating a low potential for germination, while 81% were swollen from isolate CZ-3. A day later germination was well underway. Two new observations for germination were added to Table 3.5: germ tube production, and advanced germination (germination with lateral branching). Thus, for all three observations an average of 25 and 72% spore germination was recorded for L-6332 and CZ-3 respectively.

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Table 3.4. Spore germination by isolate after 24-hours.

Isolate	Replicate	Swollen	Non-germinating	Total
L-6332	1	17	119	136
L-6332	2	20	187	207
L-6332	3	5	57	62
	Total	42	363	405
	Percent	10%	90%	
CZ-3	1	101	14	115
CZ-3	2	40	12	52
CZ-3	3	3	7	10
	Total	144	33	177
	Percent	81%	19%	

Table 3.5 Spore germination by isolate after 48-hours.

Isolate	Replicate	Swollen	Germ Tube	Advanced Germination	Non-germinating	Total
L-6332	1	18	4	34	80	136
L-6332	2	21	7	52	127	207
L-6332	3	5	1	2	54	62
	Total	44	12	88	261	405
	Percent	11%	3%	22%	64%	
CZ-3	1	8	1	99	7	115
CZ-3	2	16	1	23	12	52
CZ-3	3	0	0	3	7	10
	Total	24	2	125	26	177
	Percent	13%	1%	71%	15%	

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DISCUSSION

The goal of this study was to establish an artificial method for inducing fruiting in cultures of *C. subvermispora*. The aeration method developed proved to be successful in producing basidiocarps for isolates L-6332 and CZ-3 but not for FP-90031-sp and ME-485. Isolates FP-90031-sp and ME-485 need to be further studied.

Growing medium played an important role in basidiocarp development and abundance. Out of all the media studied 1% malt agar proved to be the most effective. Isolates L-6332 and CZ-3 consistently fruited extensively across the surface of the agar. Isolate CZ-3 responded the fastest on MA1% and produced fruiting bodies after 15 days. The Norway Spruce infusion agar with 0.15% glucose (NSA 0.15) which was used to emulate a wood substrate failed to induce basidiocarp development. The success in obtaining fruiting with isolates L-6332 and CZ-3 initiated further studies.

Overall, aeration and high humidity seems to be the main factor affecting fruiting within isolates L-6332 and CZ-3. This was not surprising since research has shown high oxygen and low carbon dioxide levels may be very important in basidiocarp formation (Wessels 1994; Rayner and Boddy 1988). Unfortunately when exposed to air movement for long periods the agar and mycelium dried up quite quickly, thus it was very important to keep the aeration racks at high moisture levels. Daily checks to make sure the towels were moist were essential. The upside-down plates and the rack cover

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trapped evaporated water moving upwards ensuring that adequate moisture was available to the fungus and to maintain the hygroscopic nature of the medium.

Although the aeration racks were successful, contamination was a re-occurring problem. Further studies using the racks should be placed in a laminar flow system which could benefit fruiting in two ways: improved air circulation, and removal of air contaminants.

The most effective method for basidiocarp development was from the jack pine boards. This was expected since the experiment closely resembles a natural situation. Although it took up to 4 months for the fungus to completely establish itself on the boards, fruiting occurred rapidly with only a one-week exposure to oxygen in the growth chambers. Furthermore, many basidiocarps would developed on one board, which could be kept growing over many months due to the large amount of substrate. However, it was quite difficult to keep the boards moist even on a daily basis. The air circulation in the germinator was enough to remove moisture from the chambers and cause some boards to dry out after a month. Once again, exposure to the air for watering and observing basidiocarp formation enabled contaminants to move inside the chambers and eventually colonize the boards. For future studies a non-air circulating chamber may be a better option.

An extremely positive result from the constant air circulation was the layers of basidiospores which coated the entire incubator. Air circulation produced from the air intake at the bottom and the fan at the top of the germinator enable spores to be circulated throughout. Small openings in the chambers may have contributed to the

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escape of some basidiospores from the chambers; however, the majority of the spores were probably released when the chambers were opened for watering and inspection. This discovery could indicate an easy method of collecting large quantities of basidiospores. Very fine filters placed at the air intake opening could trap billions of spores on a daily basis for application in biotechnology or other research.

Spore germination and production distinctly highlighted isolate CZ-3 as a more effective isolate compared to L-6332. Spore germination for CZ-3 after 48-hours was 72% and for L-6332 25%. Although this method of measuring spore germination was very basic, it does indicate a major difference between the two isolates. Spore production was also very one-sided in that CZ-3 produced a massive number of basidiospores over a 24-hour period from basidiocarps <1cm in diameter. L-6332 had a lower production rate than CZ-3, and at 25% germination was less viable. The reasons for this are unknown.

Basidiospore production in these studies was very difficult to establish accurately because of the lack of adequate containment. Very little research has been done on measuring spore production. Kramer (1982) discovered that production, release and dispersal of basidiospores were affected by a variety of environmental conditions such as temperature, relative humidity, and time of day. The method used in this paper did not specifically examine these conditions. Furthermore, isolating the two identical basidiocarps from the two different isolates was next to impossible. Surface area was taken into account by using set plug sizes, as well as the thickness of the basidiocarp. A step further would be to count the number of pores and estimate the spore production per pore. Many factors must be taken into account for exact and precise measurements of

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spore production. The methods used in this paper were used to determine if there was a basic difference between the two isolates. A possible explanation for the differences between the isolates may be attributed to the geographic locations in which the isolates were obtained. The conditions at 27°C may have favoured CZ-3 isolated from Port Townsend, WA over L-6332 isolated from Roosevelt National Forest, CO.

The potential application of CZ-3 basidiospores to large chip piles is quite feasible. A very small (0.8cm) diameter plug of basidiocarp with a surface area of approximately 0.5cm^2 has the potential to produce 1,600,000 spores over a 24-hour period. At 72% germination this amounts to 1,152,000 viable spores for pulp chip colonization. Hypothetically, a basidiocarp the size of an 8.8cm diameter petri dish would have a surface area of approximately 61cm^2 which could potentially produce 140.54 billion viable spores over a 24-hour period. The amount of pulp chips that are being produced by a mill on a daily basis would also be in the billions. Examining the amount of chips in a 1-litre container, I found that there were approximately 300 pulp chips. Thus, a volume of 1m^3 would contain approximately 300,000 pulp chips. If a typical conical chip pile were 40m in diameter and 25m high it would have a volume of 10,472m^3 and contain 3.14 billion pulp chips. Applying this volume to the spore production of a basidiocarp with the surface area of a petri dish, there would be approximately 45 spores per pulp chip after 24-hours.

Recently Saxena *et al.* (2001) published a report on efforts to induce mass sporulation of chlamydospores in *C. subvermispora* for potential use in biopulping. Although chlamydospores are a very hardy source of inoculum the process to isolate the spores takes appoximately three days to achieve. The calculations shown above clearly

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portray that basidiospore production provides the highest potential production and perhaps the most cost-effective means for large-scale inoculation of pulp chips.

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CHAPTER 4

TESTS FOR DETERMINING INCOMPATIBILITY PATTERN

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INTRODUCTION

The formation of basidiocarps allowed for the testing of factors for incompatibility. At the outset, *C. subvermispora* was assumed to be heterothallic and potentially bipolar (Nobles *et al.* 1957; Domanski 1969; Stalpers 1978; Nakasone 1981). Heterothallism is associated with a homogenic incompatibility system in which "like" mating types are incompatible and unlike mating types are compatible and produce dikaryons (Esser 1966; Raper 1966a). This allows for the development of a fruiting body through sexual compatibility between two hyphae each with haploid nuclei (Raper 1966a). Two different categories of homogenic sexual incompatibility are referred to as being bipolar or tetrapolar (Esser 1966) and are found only in the class Homobasidiomycetes (Casselton and Kues 1994). The bipolar system has a single factorial allele at one locus (A) and is usually represented with different factors such as A1, A2, A3 . . . A26 etc. (Raper 1966a; Raper 1966b; Esser 1966; Casselton and Kues 1994). When two numbered "A" factors are alike and are crossed, such as A1 x A1 or A2 x A2, incompatibility is the result; however, combinations such as A1 x A2 result in successful mating as a consequence of reciprocal transfer of nuclei and dikaryotization. Dikaryotization can be judged microscopically by the formation of clamp connections at septa. Tetrapolar systems have two loci (A and B) with several alleles for compatibility. The "A" locus is involved with clamp formation and synchronized nuclear division; and locus "B" is responsible for coordinating nuclear migration and fusion of the hook cell

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of the clamp (Raper 1966a; Raper 1966b; Esser 1966; Casselton and Kues 1994). This system produces four different reactions as follows:

1. Uncommon-AB crosses (compatible). Here there are two possible crosses: A1B2 x A2B1 and A1B1 x A2B2. Migration of nuclei occurs first followed by conjugate division and clamp formation. True clamp (hook cell) connections are formed with complete fusion to the hyphal wall. Dikaryotic cells are established.
2. Common-A/uncommon-B crosses (Hemicompatible A). Two possible combinations are produced: A1B2 x A1B1 and A2B1 x A2B2. Nuclear division and clamp formation do not occur; however, the exchange of nuclei between hyphae and nuclear migration are active.
3. Common-B/uncommon-A crosses (Hemicompatible B). Two possible combinations are found: A1B2 x A2B2 and A2B1 x A1B1. Nuclear division and the exchange of nuclei occur regularly. The occurrence of clamp cell formation (pseudoclamps) takes place; however, there is no fusion of clamp cells and thus no nuclear migration.
4. Common-AB crosses (Incompatible). Here there are four potential combinations: A1B2 x A1B2, A2B2 x A2B2, A1B1 x A1B1, and A2B1 x A2B1. The exchange of nuclei is sparse and restricted to the contact zone. Nuclear division, clamp cell formation and nuclear migration are non-operational (Esser 1966; Raper 1966a; and Casselton and Kues 1994).

Some of the best-detailed work on the sexuality of Hymenomycetes occurred in the 1950s through 70s. Aschan (1954) reported incompatibility behaviour in the edible mushroom *Collybia velutipes*. Importantly, she examined the hyphae for clamps on both sides of the contact zone instead of just the narrow zone of contact. By using this more thorough approach, *C. velutipes* which had been thought to be bipolar, was found to be tetrapolar. Takemaru (1961) confirmed Aschan's work on *C. velutipes* and used macroscopic characteristics of the hyphal mat, incomplete dicaryotization and unilateral compatibility to present a tetrapolar scheme with various anomalies. Further work by Takemaru (1961) examined the mating systems of *Coprinus marcorhizus, Coprinus rostrupianus, Lentinus edodes, Panus tigrinus, Pleurotus spodoleueus, Psilocybe coprophila* and *Schizophyllum commune*. Later, in 1966 detailed work by Raper with *S.*

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commune formed the basis of his book Genetics of Sexuality in the Higher Fungi. Raper, his students, and others have made considerable advances since this time. Ginns (1974) examined pairing tables from several authors on various Hymenomycetes which showed a bipolar pattern. One problem with these tables was the misinterpretation of the numerous failures for expected compatible matings to produce the clamps. Aberrant pairings within groups could be split up and re-categorized, and by doing so, Ginns (1974) was able to show a tetrapolar interpretation of the reactions. However, Ginns (1974) failed to speculate on the reasons for the anomalous results.

The purpose of this chapter is to investigate the sexuality of *C. subvermispora*. The methods and materials for determining heterothallism, single spore isolation and the tests for interfertility are presented as well as the results for determining heterothallism and the tests for interfertility. A discussion of the implication of these results follows.

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METHODS

Nuclear Complement Determination

The nuclei of spores and mycelium of *C. subvermispora* were stained using a Giemsa Stain solution (EM Science®). Spores and mycelium were fixed for two minutes using Carnoy's fixative consisting of 1 part HAc: 3 parts 95% ethyl alcohol. The fixed spores and hyphae were hydrolyzed in 5N HCl for 5 minutes and then were rinsed with water. A solution of 1:1 Giemsa stain and H_2O were applied to the spores and hyphae and left in moist conditions for 24-hours. Examination of the nuclei required the use of a phase contrast light microscope at 1000x magnification. The photographs were taken with a Nikon™ digital camera mounted on a Nikon Eclipse E400 compound microscope.

Single Spore Isolation

Fruiting bodies of CZ-3 that formed on 1% malt extract agar plates were used as the source for spores. An agar plug with the basidiocarp was removed and inverted from the top petri dish lid with Vaseline®. The lid was then placed over a 1% malt agar dish and spores were allowed to fall on the agar surface for approximately 5 minutes. The approximate area where the spores fell was circumscribed on the bottom of the petri dish and then the lid was rotated to another location on the agar and left for 10 minutes. This process was repeated until five spore prints were established on the agar at increasing intervals of 5 minutes.

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Under a high powered dissecting scope, widely spaced spores were located on the surface of the agar. Using an extremely thin dissecting needle, single spores were carefully removed with the medium and placed on a plate of 1% malt agar using sterile technique. Solitary spores that traveled far away from the main spore grouping were the easiest to isolate. A total of 35 spores were transferred to germinate on the plates. Spores that germinated and produced colonies were transferred to agar tube slants of 1% malt agar. Mycelium from each of 15 tubes was then re-plated on 1% malt agar and examined for non-clamped monokaryotic hyphae. Fifteen single spore isolates were labeled A-O and were found to be simple septate except for isolate F, which was clamped (dikaryotic). Isolates were maintained in a refrigerator until needed for study.

Test for Incompatibility

The 14 single spores were crossed in all combinations for a total of 91 unique crosses. All crosses were examined for clamp connections and pseudoclamps and the frequency of both characteristics. In addition, macroscopic features and any other distinguishing features were also observed and recorded. Appendix II lists detailed observations for each cross.

Single spore crosses were organized in four pairing tables: mating response showing bipolarity and tetrapolarity with the presence of complete clamp connections in the zone of contact; mating response between the zone of contact and on both sides of each inoculation points; and phenotypic (macroscopic) response (barrage or flat) of the mycelium.

Crosses between all possible combinations of single spore isolates were taken two at a time as described by Aschan (1954), Takemaru (1961) and Setliff (1970) and were paired as follows. A piece of agar containing the monosporous mycelium was

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placed left of center on 1% malt agar plates. A second isolate was placed right of center 2-3 cm away from the first isolate. The paired crosses were incubated at 27°C for 2-weeks and then examined microscopically. Mycelium was taken from the zone of convergence and on either side of the contact zone. The mycelium was stained in phloxine and examined for the presence (+) or absence (-) of clamp connections. In addition, pseudoclamps and clamp frequency were recorded. Pseudoclamps were characterized microscopically by examining hook cell fusion. If the hook cells did not fuse with the hyphae or curl to the side of the hyphae, a common-B interaction was likely occurring (Raper 1966a; Casselton and Kues 1994). In the case of this experiment many clamps per mating were observed very closely before they were classified as pseudoclamps. Clamp frequency was determined qualitatively and labeled high, few, moderate and patchy (see Appendix II).

Pairing tables were organized in a grid format in order to map the allelic configuration of the crosses. Table organization and categorization began by following Ginns' (1974) work to group similar responses together in order to produce the lowest number of failures among each grouping. In addition, crosses were grouped with regard to unilateral compatibility (Setliff 1970; Ginns 1974; Takemaru 1961) or incomplete dicaryotization (Aschan 1954; Takemaru 1961). Both unilateral and incomplete compatibilities are described as non-dicaryotization or "illegitimate" pairings (Raper 1966a; Aschan-Aberg 1960b; Aschan 1954). Unilateral compatibility is represented by clamps in the contact zone and on only one side (Raper 1966a; Takmaru 1961), whereas incomplete compatibility is restricted to clamps only in the zone of contact and absent on either side (Aschan 1954; Takemaru 1961). Finally, macroscopic observations were also used to categorize the crosses (Takemaru 1961). Phenotypic characterization of the

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crosses followed work by Raper (1966) in that a zone of appressed mycelium lacking aerial hyphae in the zone of convergence was termed “flat” and a thick discernible zone of aerial hyphae in the area of contact was deemed a “barrage zone”.

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RESULTS

Nuclear Complement Determination

The Giemsa stain was successful in staining the nuclei of both the spores and the hyphae. Figure 4.1 consists of spores photographed at 1000x magnification showing uninucleate or haploid spores. Many spores were examined and all were found to be uninucleate. Figure 4.2 shows various dikaryotic hyphae with pairs of stained nuclei in the stock cultures of isolate CZ-3. The hyphae were typically dikaryotic.

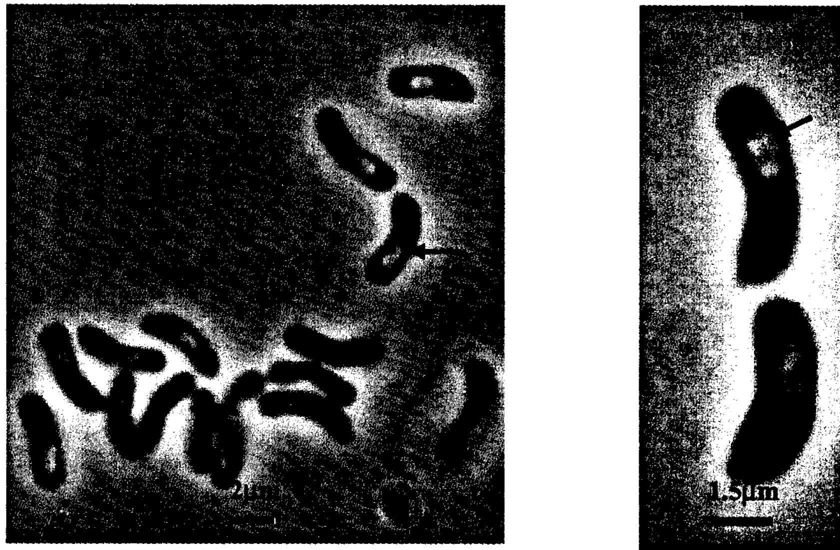

Figure 4.1. Giemsa stained basidiospores of *C. subvermispora* with single nuclei. Spores were photographed under phase contrast at 1000x final maginfication. The light areas inside the spores (shown with the arrows) are the single nuclei.

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Figure 4.2. Giemsa stained dikaryotic hyphae of *C. subvermipora*. Hyphae were photographed under bright field. The arrows indicate the nuclei stained dark purple inside the hyphae which are occurring in haploid pairs.

Test for Incompatibility

The interpretation of the mating system as being either bipolar or tetrapolar depends upon the approach used in recording the data. Table 4.1 shows the mating reactions to be essentially bipolar when only the zone of contact was examined for clamp connections. Counting the presence of pseudoclamps as being indicative of mating, the table can be organized in such a manner that shows two distinct allelic responses: presence and absence of clamps with an unexpected failure rate of 2% in the one failed cross K x J. However, pseudoclamps do not represent complete dicaryotization and so if these are discounted, along with the negative reactions, as part of the expected positive matings, the failure rate then becomes 26/49 or 53% error. Only one mating in the table that was expected to be positive was persistently negative.

Based upon reciprocal positive pairing results a tetrapolar tendancy is apparent.

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A reinterpretation of Table 4.1 in terms of pseudoclamp formation and complete

Table 4.1 Single spore mating reactions of *C. subvermispora* showing clamps found only in the contact zone. The pattern follows a bipolar system, where "+" represents presence of clamps, "⊥" shows pseudoclamps and "-" indicates absence of clamps.

		A1							A2						
		E	J	B	L	D	C	M	N	K	0	I	G	H	A
A1	E		-	-	-	-	-	-	⊥	⊥	⊥	⊥	⊥	+	+
	J	-		-	-	-	-	-	⊥	-	⊥	⊥	⊥	+	+
	B	-	-		-	-	-	-	⊥	⊥	⊥	⊥	⊥	+	+
	L	-	-	-		-	-	-	⊥	⊥	⊥	⊥	⊥	+	+
	D	-	-	-	-		-	-	+	+	+	+	+	⊥	⊥
	C	-	-	-	-	-		-	+	+	+	+	+	⊥	⊥
	M	-	-	-	-	-	-		+	+	+	+	+	⊥	⊥
A2	N	⊥	⊥	⊥	⊥	+	+	+		-	-	-	-	-	-
	K	⊥	-	⊥	⊥	+	+	+	-		-	-	-	-	-
	O	⊥	⊥	⊥	⊥	+	+	+	-	-		-	-	-	-
	I	⊥	⊥	⊥	⊥	+	+	+	-	-	-		-	-	-
	G	⊥	⊥	⊥	⊥	+	+	+	-	-	-	-		-	-
	H	+	+	+	+	⊥	⊥	⊥	-	-	-	-	-		-
	A	+	+	+	+	⊥	⊥	⊥	-	-	-	-	-	-	

dikaryotization is shown in Table 4.2. An obvious tetrapolar pattern arises showing uncommon-A/common-B groupings producing pseudoclamps and the compatible matings yielding true clamp formations. Using the Ginns (1974) method for error rate, 23 out of 23 compatible matings were successfully grouped producing a failure rate of 0%. Figure 4.3 displays typical pseudoclamps found in a common-B interaction as compared to a true clamp.

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Figure 4.2. Single spore mating reactions of *C. subvermispora* showing terapolarity where "⊥" represents pseudoclamp formation (common-B reaction), and "+" indicates dicaryotization ("true" clamp formation = uncommon-AB reaction).

		A1B2				A2B2					A1B1			A2B1	
		E	J	B	L	N	K	O	I	G	D	C	M	H	A
A1B2	E		-	-	-	⊥	⊥	⊥	⊥	⊥	-	-	-	+	+
	J	-		-	-	⊥	-	⊥	⊥	⊥	-	-	-	+	+
	B	-	-		-	⊥	⊥	⊥	⊥	⊥	-	-	-	+	+
	L	-	-	-		⊥	⊥	⊥	⊥	⊥	-	-	-	+	+
A2B2	N	⊥	⊥	⊥	⊥		-	-	-	-	+	+	+	-	-
	K	⊥	-	⊥	⊥	-		-	-	-	+	+	+	-	-
	O	⊥	⊥	⊥	⊥	-	-		-	-	+	+	+	-	-
	I	⊥	⊥	⊥	⊥	-	-	-		-	+	+	+	-	-
	G	⊥	⊥	⊥	⊥	-	-	-	-		+	+	+	-	-
A1B1	D	-	-	-	-	+	+	+	+	+		-	-	⊥	⊥
	C	-	-	-	-	+	+	+	+	+	-		-	⊥	⊥
	M	-	-	-	-	+	+	+	+	+	-	-		⊥	⊥
A2B1	H	+	+	+	+	-	-	-	-	-	⊥	⊥	⊥		-
	A	+	+	+	+	-	-	-	-	-	⊥	⊥	⊥	-	

Observation of both sides of the matings, away from the contact zone, resulted in additional results and changes in Tables 4.1 and 4.2. Unilateral and incomplete compatibility were observed with both complete clamp formation and pseudoclamp formation.

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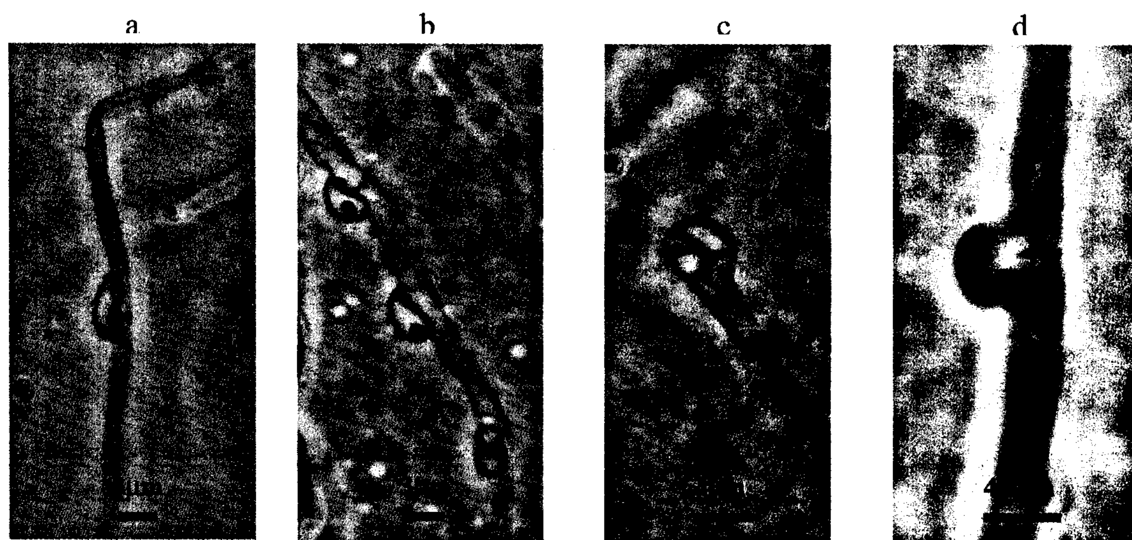

Figure 4.3. Various pseudoclamps (a,b and c) from a uncommon-A/common-B interaction compared to a true clamp (d) from a uncommon-AB interaction. Notice the hook cell fusion in photograph (d) compared to the non-fused hook cells produced by the pseudoclamps.

Complete or bilateral dicaryotization, where clamps were found in all three locations, was also recorded. Grouping and reorganization of the crosses involved the separation of all three genotypic characteristics into individual groups as shown in Table 4.3. Although each allelic grouping does not contain expected homogeneous characteristics, a definite pattern was produced that is essentially that of Table 4.2.

Allelic factors shown in Table 4.3 were determined according to the most common reaction involved within each group. Unilateral "⊥" and incomplete "(⊥)" pseudoclamp formation are classified as an uncommon-A and common-B pairing (Raper 1966a; Takemaru 1961; Aschan 1954). Grouping A1B2 x A2B2 has a mixture of mostly unilateral and incomplete reactions and thus can be classified as such an allelic configuration. Furthermore, grouping A2B1x A1B1 can be classified as an incomplete reaction and thus labeled appropriately.

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Table 4.3. Single-spore mating reactions of *C. subvermispora* observing both sides of the contact zone indicating a tetrapolar system. "(+)", clamps found only in zone of contact; "+", clamps found in contact zone and on both sides of the contact zone; "+" clamps found on one side of the contact zone (unilateral compatibility); "(⊥)", pseudoclamps found only in the zone of contact; "⊥", pseudoclamps found in contact zone and on both sides of the contact zone; "⊥" pseudoclamps found on one side of the contact zone.

		A1B2				A2B2					A1B1			A2B1	
		E	J	B	L	N	K	O	I	G	D	C	M	H	A
A1B2	E		-	-	-	⊥	⊥	(⊥)	(⊥)	⊥	-	-	-	+	+
	J	-		-	-	⊥	-	(⊥)	(⊥)	(⊥)	-	-	-	+	+
	B	-	-		-	⊥	(⊥)	(⊥)	(⊥)	⊥	-	-	-	+	+
	L	-	-	-		⊥	⊥	⊥	⊥	⊥	-	-	-	+	+
A2B2	N	⊥	⊥	⊥	⊥		-	-	-	-	+	+	+	-	-
	K	⊥	-	(⊥)	⊥	-		-	-	-	+	+	(+)	-	-
	O	(⊥)	(⊥)	(⊥)	⊥	-	-		-	-	+	+	(+)	-	-
	I	(⊥)	(⊥)	(⊥)	⊥	-	-	-		-	+	+	+	-	-
	G	⊥	(⊥)	⊥	⊥	-	-	-	-		+	+	+	-	-
A1B1	D	-	-	-	-	+	+	+	+	+		-	-	(⊥)	(⊥)
	C	-	-	-	-	+	+	+	+	+	-		-	(⊥)	(⊥)
	M	-	-	-	-	+	(+)	(+)	+	+	-	-		(⊥)	⊥
A2B1	H	+	+	+	+	-	-	-	-	-	(⊥)	(⊥)	(⊥)		-
	A	+	+	+	+	-	-	-	-	-	(⊥)	(⊥)	⊥	-	

Uncommon-AB interactions are classified as compatible mating where complete dicaryotization "+" occurs (Raper 1966). Table 4.3 illustrates the two groupings

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classified within this interaction. Grouping A1B1 x A2B2 indicates 11 out of 15 crosses with complete compatibility, while grouping A2B1 x A1B2 resulted in 5 of 8 crosses. If incomplete dikaryotization "(+)" is considered an error in the compatible matings the failure rate is approximately 9%.

Finally, common-A and common-AB interactions do not produce clamps and are therefore classified as negative (Raper 1966). Differentiation between the two interactions occurs once the clamped groupings are established and labeled.

The macroscopic response of the single spore crosses produced varied results with a majority of matings following an expected pattern (Table 4.4). Table 4.5 indicates the percentage of each phenotypic response by mating types. Common-AB and Common-A/uncommon-B interactions produced both a flat reaction and no reaction. Uncommon-AB pairings exhibited barrage zones (61%) and no reaction (35%), while uncommon-A/common-B crosses displayed barrage (73%) and flat responses (23%). A consistent pattern was noted where unilateral pseudoclamps were 100% unambiguous for the barrage effect.

True clamp and pseudoclamp frequency in the zone of contact and on either side of the inoculation points was recorded in Appendix II. Uncommon-A/common-B matings produced mainly moderate pseudoclamp formation. All pairings where pseudoclamps were found strictly in the zone of contact had a moderate frequency of clamp formation, which were mostly aberrant (not occurring at each septum). Mating D x A was the only pairing that was observed to produced very few false-clamps. Uncommon-AB crosses were observed, for the most part, with high frequencies of true clamp formation consisting of both normal (occurring at each septum) and aberrant patterns. Crosses O x M and M x K produced limited dikaryotization found only in the

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zone of contact. The frequency of clamp formation with these two pairings was observed to be more moderate than high. Matings where complete dicaryotization occurred mainly contrived high frequencies of clamp formation; however, five crosses were observed having moderate to few clamps on one side.

Table 4.4. Single-spore mating reactions of *C. subvermispora* labeled according to the marcoscopic characteristics of the mycelial mat. "B", barrage zone; "F", flat; "+" and "-", no observable reaction.

		A1B2				A2B2					A1B1			A2B1	
		E	J	B	L	N	K	O	I	G	D	C	M	H	A
A1B2	E		-	F	-	B	B	B	F	B	F	F	F	B	+
	J	-		F	F	B	F	B	B	F	F	-	-	B	+
	B	F	F		F	B	B	F	F	B	F	F	F	+	B
	L	-	F	F		B	B	B	B	B	F	-	F	B	B
A2B2	N	B	B	B	B		F	F	F	-	B	+	B	-	F
	K	B	F	B	B	F		F	F	F	B	B	B	-	F
	O	B	B	F	B	F	F		F	F	B	B	F	-	F
	I	F	B	F	B	F	F	F		F	B	+	+	-	-
	G	B	F	B	B	-	F	F	F		B	+	+	-	-
A1B1	D	F	F	F	F	B	B	B	B	B		F	-	B	+
	C	F	-	F	-	+	B	B	+	+	F		F	+	F
	M	F	-	F	F	B	B	F	+	+	-	F		B	B
A2B1	H	B	B	+	B	-	-	-	-	-	B	+	B		F
	A	+	+	B	B	F	F	F	-	-	+	F	B	F	

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Table 4.5. Percentage of three macroscopic characteristics of the mycelial mat for various matings of *C. subvermispora.*

Mating	Barrage	Flat	No Reaction
Uncommon-A/common-B	73%	23%	4%
Uncommon-AB	61%	4%	35%
Common-A/uncommon-B	0%	55%	45%
Common-AB	0%	80%	20%

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DISCUSSION

According to Nakasone (1981), Nobles *et al.* (1957) referred to *C. subvermispora* as *Poria notata* Overh. Nobles *et al.* (1957) determined the fungus to be heterothallic with a bipolar incompatibility system. Later, Domanski (1969), Stalpers (1978) and Nakasone (1981) confirmed these observations. Without question *C. subvermispora* is heterothallic; however, further investigation shows the fungus to possess tetrapolar tendencies as shown by the presence of pseudoclamps in many of the crosses. Furthermore, examination of both sides of the contact zone established unilateral compatibility and incomplete dicaryotization as a common occurrence with *C. subvermispora*. The presence of pseudoclamps was a very important characteristic in the mapping of the allelic patterns. It was this mapping system which showed a clear pattern of tetrapolarity with the fungus. Confirmation of these results with another isolate of *C. subvermispora* is recommended for future research.

Unilateral and incomplete compatibility groupings were organized in the best possible manner with the amount of isolates that were examined. A larger grouping of single spore isolates may have produced a much clearer pattern; however, the essential goal of determining the sexuality of *C. subvermispora* was achieved. By following Ginns' (1974) interpretation of pairing tables, there is very little error within the groupings. The K x J cross failed to produce clamps or pseudoclamps and is the only aberrant cross within the allelic groups in terms of clamp formation. Two more potential

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errors were found in the compatible matings. Incomplete dikaryotization occurred in crosses K x M and O x M suggesting a common-B rather than an uncommon-AB interaction. It is the B factor that controls nuclear migration and so clamps must be found beyond the zone of contact in order to be considered a complete mating. This was not the case with both crosses; however, there is the possibility that the nuclear migration is delayed and clamps will form after a long period of time.

The percentage of macroscopic traits among the paired matings was comparable with studies done by Raper (1966) and Takemaru (1961). Raper categorized the common-A interactions as a typical flat reaction although sometimes producing no observable reaction. The common-B interaction was categorized for the production of a barrage zone; however, in some cases no intermycelial reaction was observed. Furthermore, common-AB pairings were classified as having no reaction. Takemaru (1961) commented that common-B matings could not be strictly categorized as barrage since observations showed barrage zones being formed in all four mating types with the fungus *C. velutipes*. Although nearly 100% of common-B crosses produced barrage zones, among the common-A and common-AB matings as high as 57% barrage zone formation was found and common-AB had a low frequency of 6%. The results for *C. subvermispora* show the majority of the common-B reactions were barrage at 73%, but 23% were flat. The flat response was a surprising observation since it is an uncharacteristic reaction for common-B matings. Uncommon-AB crossings of *C. subvermispora* were similar to Takemaru's findings with observations mainly split between barrage and no reaction. As expected common-A crossings depicted a flat response for the majority of the matings; however, 45% portrayed no observable reaction. Finally, another surprising trait of *C. subvermispora* was the 80% frequency of

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distinct flat regions among the common-AB matings with only 20% found to have no reaction.

Variation among the groupings for macroscopic observations could have been attributed to the growing medium. The 1% malt agar used for the spore crosses did not produce very much aerial hyphae and thus distinction between barrage zones and no reaction was difficult to determine for certain. However, the flat reactions were easily identified with this medium. Higher nutrient concentrations such as 3% malt and 3% potato dextrose agar should be used in future studies because they cause more aerial hyphae to form.

Clamp distribution, frequency and arrangement among the various hyphal mating systems for *C. subvermispora* were similar to findings by Raper (1966) and Takemaru (1961). They found common-A and common-AB pairings are strictly simple septate and this coincides with the findings for *C. subvermispora.*

Common-B matings of *C. subvermispora* produced three different pseudoclamp distributions and at varied frequencies and arrangements. Pseudoclamp distribution occurred strictly in the zone of contact, unilaterally and bilaterally. Pseudoclamps were moderately produced when found strictly in the zone of contact and typically followed an aberrant arrangement. Pseudoclamps located in a unilateral distribution were found to develop sparse to moderate false-clamps that occurred in irregular arrangement. When the false-clamps were distributed on both sides of the contact zone they were extremely difficult to find and highly unorganized in arrangement. Takemaru (1961) indicated common-B matings with *C. velutipes* produced both unilateral and limited compatibility. In both cases clamp formation was either abundant or limited and followed both normal and aberrant formation. Similar observations were found with *C.*

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subvermispora. Also, Raper's (1966a) common-B observations with *S. commune* showed unilateral compatibility with pseudoclamps that formed randomly at septations while others developed without septations. Takemaru (1961) explained the occurrence of both unilateral and limited compatibility with a common-B interaction from the same fungus. The findings suggest that mating types could be grouped in two behavioural categories: donor and acceptor mycelium. Donor mycelium is a "monokaryon which can donate its dikaryotizing nucleus to a mate but accepts none from the mate" and acceptor mycelium will accept the nucleus from the mate (Takemaru 1961). The following indicates the clamp-forming reactions with both acceptor and donor mating types as determined by Takemaru (1961):

- When two uncommon-AB acceptor pairings occur, complete compatibility occurs;
- When two common-B acceptor pairings are crossed, contact zone heterokaryotization (limited compatibility) is observed;
- When an uncommon-AB acceptor is crossed with an uncommon-AB donor, unilateral dikaryotization occurs;
- When a common-B acceptor is crossed with a common-B donor, contact zone heterokaryotization may develop or no clamps are formed;
- Any common-A or common-AB whether acceptor or donor do not form clamps;
- Any donor crossed with another donor regardless of the mating type will not produce clamps.

Although this provides an explanation for both contact zone compatibility and unilateral compatibility, it does not explain the occurrence of pseudoclamps in all three locations. This phenomenon may arise from common-B acceptor crosses that are not restricted to the contact zone and can form pseudoclamps bilaterally. Bilateral pseudoclamp formation is a newly discovered phenomenon and should be examined in greater detail.

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Uncommon-AB matings were characterized by the presence of true clamp formation. Although the majority of the matings were bilateral some responses were unilateral and restricted to the contact zone. Another uncharacteristic finding was with uncommon-AB matings. Observations by both Raper (1966) and Takemaru (1961) suggest that with uncommon-AB crosses clamps are true and always normally arranged occurring at each septum. It was found that although such crosses produced true clamps, some hyphae showed an aberrant arrangement of clamps while others seemed to have clamps at each septum. The cause of this peculiarity is not yet known and requires further examination of the hyphae for interference factors of some kind that alters genetic and phenotypic expression.

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CHAPTER 5

PRODUCTION OF EXTRACELLULAR OXIDASE

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INTRODUCTION

The extracellular ligninolytic enzymes produced by *C. subvermispora* are manganese peroxidase (MnP) and laccase (Ruttimann *et al.* 1992a; Ruttimann *et al.* 1992b; Ruttimann-Johnson *et al.* 1993; Tuor *et al.* 1995; Karahanian *et al.* 1998; Leonowicz *et al.* 1999). The optimization of these enzymes in cultural conditions has been researched intensively for the past 10 years due to their potential applications to the biopulping industry. Niku-Paavola *et al.* (1990) discovered that different cultural conditions affected the production of ligninolytic enzymes in the white-rot fungus *Phlebia radiata*. Interestingly, laccase and MnP were increased when the fungus grew on wood-based media. Ruttimann *et al.* (1992a) reported that among five different isolates of *C. subvermispora*, the production of MnP increased when nitrogen levels were increased at different levels in the growing media. Ruttimann-Johnson *et al.* (1993) and Tapia and Vicuna (1995) found different levels of nitrogen and glucose influence enzyme production in liquid cultures. Increased levels of nitrogen were beneficial to enzyme production, similarly enzyme production was increased with cultures containing 1% glucose and decreased at a lower level (0.1% glucose). Fukushima and Kirk (1994) discovered that the laccase activity of *C. subvermispora* was optimal at a broad range of pH levels between 3 to 5. Lobos *et al.* (2001) theorized that the different enzyme activities associated with various isolates of *C. subvermispora* on different media could be associated with the natural linkages of particular substrates with certain isolates. The term "natural linkages" suggests that the chemistry of certain

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media, developed in culture, can closely resemble the natural substrates upon which the isolates grow and produce reactions similar to those found in a natural environment.

There are many cultural tests that can be utilized for detecting extracellular enzyme activity with white and brown rot fungi. Among these tests the gum guaiac test for extracellular laccase and tyronsinase developed by Nobles (1958a) is faster (Rayner and Boddy 1988). Although the gum guaiac reaction does not distinguish between the two enzymes, it is one of the more commonly used tests in cultural studies (Marr 1979). Nobles (1958a) found the chemical solution to be 85% in agreement when compared to the Bavendamm method (Bavendamm 1928). Stalpers (1978) used α-naphthol, p-cresol and other chemicals to detect extracellular oxidases and found that *C. subvermispora* produced three polyphenol enzymes, *viz*. laccase, tyrosinase and peroxidase. Stalpers (1978) allowed the test to run for 3 days and noted results at 24, 48 and 72 hours.

Lombard and Gilbertson (1965) reported four white-rot fungi under the genus *Poria* that produced negative or faint oxidase reactions growing on a medium of 2.5% diamalt agar using both the gum guaiac and Bavendamm tests. Preliminary results with *C. subvermispora* showed it to be in this same category. Further preliminary tests with gum guaiac indicated that type of medium and sugar levels were instrumental in the amount of extracellular oxidases produced. The purpose of this chapter is to report on the production of extracellular oxidases by *C. subvermispora* when grown on various media. A method for quantifying the intensity of gum guaiac reactions on different media was developed with image analysis software. A discussion of the implications of the results follows.

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MATERIALS AND METHODS

Presence/absence of extracellular oxidase by medium and isolate

Nobles (1958a) developed a quick and effective method of testing for extracellular enzyme laccase and tyrosinase using an alcoholic gum guaiac solution. The solution consists of 0.5g gum guaiac in 30 mL of 95% alcohol. The solution is a pale brown colour and turns blue after 2-3 minutes when applied directly to the mycelium with either laccase and/or tyrosinase (Nobles 1958a).

Preliminary tests with the gum guaiac solution showed no reaction with cultures of CZ-3 and L-6332 one-month in age that were growing on MA1%, MA3%, PDA, CSY, and CS0.5. However, when the solution was applied to mycelium that had grown on a jack pine board for 3 months, it instantly turned blue. A Norway spruce infusion agar (NSA) was made, as described in chapter 1, to emulate a natural substrate for the fungus. To test the impact that sugar levels have on the extracellular oxidase reaction, different concentrations of glucose were added to the infusion agar. Preliminary observations with cultures of *C. subvermispora* grown on NSA media containing glucose levels of 0.15, 0.3, 0.6 and 1.2% indicated that the different levels had an impact on the intensity of the gum guaiac reaction and the speed of the reaction. Ten media were used in the gum guaiac experiment *viz.* MA1%, MA3%, PDA, CSY, CS0.5 and NSA; as well as, the NSA with the four glucose levels.

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Two time periods were examined using cultures grown for one-and-half weeks (11 days) and three weeks (21 days) to test the effect of culture age on the production of extracellular oxidase.

The intensity of the blue reaction was measured by image analysis of colouration. It was important to have as close to true colours as possible in order to adequately represent the intensity of the reaction. Tungsten light bulbs (3200K, 500W, 120V) were used along with Kodak Professional Porta™ 100T Tungsten film ASA 64. This allows for near perfect colour balance between the true colours present and the film (Kodak 1981). In order to account for any shift in colour during the film development, a colour chart was included in each picture and the pictures were developed according to the colours on the original chart.

The experiment was set up in the following manner. A typical camera stand and table were set up so photos of the petri dishes could be taken from directly above (see Figure 5.1). Two Tungsten lights were aligned at a 45° to the table surface. Each petri dish, along with the colour chart, was centered in the field of view of the camera. The exposure time for the camera was set for 1/30 second at f8.3. Once in place, the dish cover was removed and two equal drops of gum guaiac were added to the mycelial surface. The reaction was timed for exactly 3 minutes the moment the drops were added to the plates. After this time, the picture was taken and the process was repeated until all the dishes were photographed (see Figure 5.2). Pictures were taken of the gum guaiac reactions on cultures grown at 11 days and 21 days on the 10 media with isolates CZ-3 and L-6332. Each treatment combination was replicated twice and photographed.

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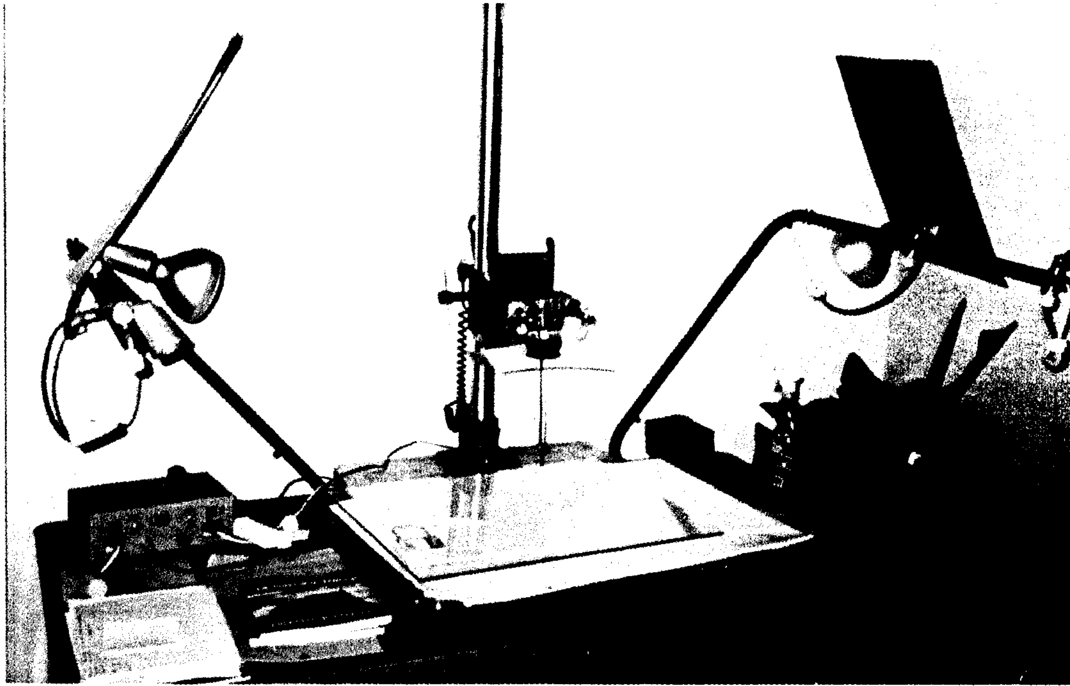

Figure 5.1. Camera stand setup for taking pictures of the gum guaiac extracellular oxidase reaction. The two tungsten light bulbs were angled at 45° and the camera was set at a height of 34.5 cm above the table.

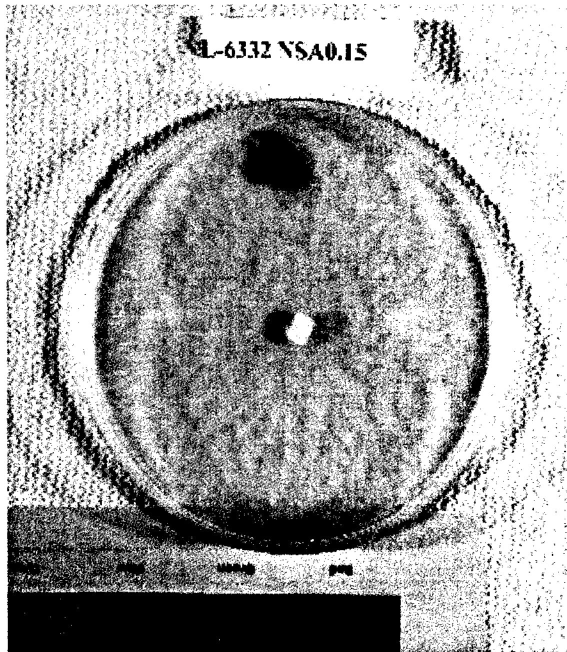

Figure 5.2. Typical picture taken of the gum guaiac reaction after 3 minutes. The dark blue stain is the extracellular oxidase reaction with the gum guaiac solution. Below is the colour chart placed in each picture.

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Once the pictures had been developed, each picture was scanned using a photo scanner at a resolution of 600 dots per inch. The colour chart on the photographs was used to calibrate each scanned image for colour balanced. Using ERDAS Imagine 8.5™ an inquiry box was established for the gray section on the colour chart for each image. Data in the inquiry box indicated the mean pixel values for the red, green and blue spectral layers. The means for each layer from each picture were recorded on a spreadsheet. From these means the image with the highest pixel values, the brightest image, was determined. Using the ERDAS™ model maker the red, green and blue pixel values for each image were adjusted according to the exact same values as the brightest scanned image. Each image was completely colour balanced and thus could be quantified for the gum guaiac reactions.

The intensity of the gum guaiac reactions was quantified by isolating the drops of gum guaiac using the ERDAS™ subset option. Each image was zoomed in until the entire viewer showed the gum guaiac areas at a magnified level. The areas were carefully cut out of the image and saved as separate subset image. It was crucial to make sure that only the gum guaiac was captured so that the analysis would not be flawed. Figure 5.3 illustrates how each gum guaiac reaction was isolated.

All pixel values inside the subset image were determined by ERDAS™ for the red, green and blue layers. These values were averaged for each subset producing a single numerical value for each layer. Appendix III (Table AIII.1) lists the average pixel values of each layer for each treatment combination.

The initial data analysis began with a Pearson Product-Moment correlation to test the correlation of the pixel values among the red, green and blue layers (Schulman

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1992). The calculations for the Pearson Product-Moment correlation, ANOVA and LSD were completed by Data Desk version 6.0.1™.

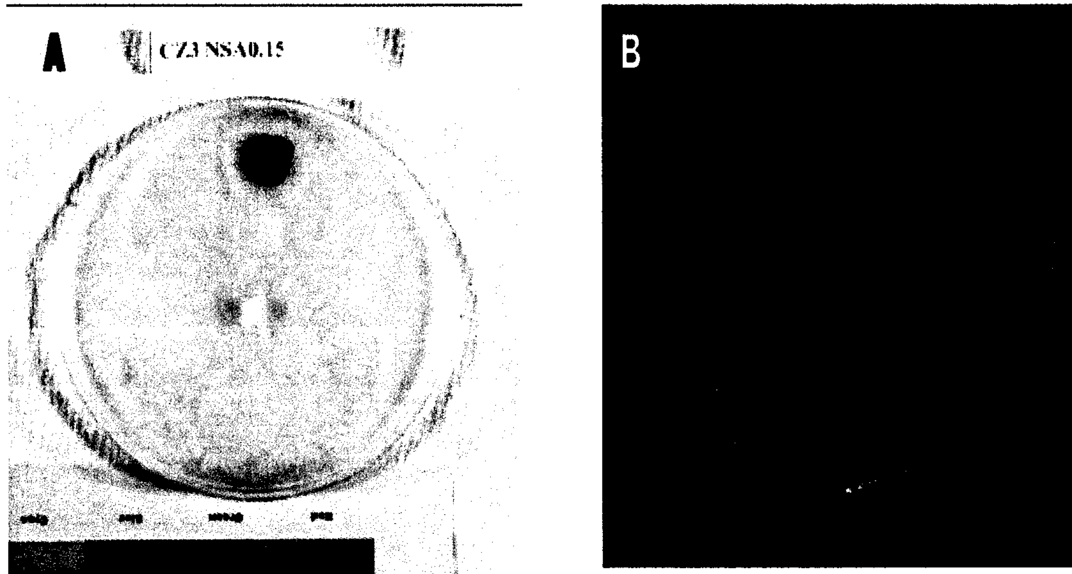

Figure 5.3. Isolation of the gum guaiac reaction with ERDAS Imagine 8.5™. Image A shows a dark blue reaction with the gum guaiac. Image B shows the isolations of the reaction as a separate image.

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RESULTS

<u>Presence/absence of extracellular oxidase by medium and isolate</u>

Initial visual observations were made in order to predict what the statistical analysis would indicate. It was evident that type of media played a role in extracellular enzyme production for *C. subvermispora*. Initial tests found that MA1% did not produce a blue reaction when the gum guaiac was applied. Knowing that the fungus produces laccase and tyrosinase, a drop was applied to mycelium growing on a jack pine board. An immediate dark blue reaction was produced indicating that it was the MA1% medium that inhibited the production of the enzyme. When the experiments were initiated on the different media, it was evident that some media may have been inhibiting the production of the enzyme. Both isolates on PDA, MA1% or CS0.15 showed no signs of a blue reaction. Isolates growing on NSA1.2 produced a faint blue reaction after 11days and on MA3% a moderate reaction was observed after the same time period. Both isolates growing on lower glucose levels of the NSA medium produced the darkest reactions (lowest pixel values) after 11 days and similarly after 21 days. The reactions after 21 days did not seem to be as intense as cultures grown for 11 days. Figure 5.4 shows typical examples of the drops of gum guaiac that were placed on the media. Some drops dispersed in a circular shape across the mycelium or remained as a circular blotch while others dispersed in irregular shapes upon contact. When in contact with the solution, the fungus produced varied intensities of the blue reaction depending on the medium. Some reactions produced a faint blue colour in areas of the gum guaiac,

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some produced a uniform blue reaction and some did not change colour at all. Figure 5.4 provides a visualization of the different intensities of the gum guaiac reactions on the different media. In order to measure the intensity of each reaction, the entire drop of gum guaiac was examined using the ERDAS™ subset option.

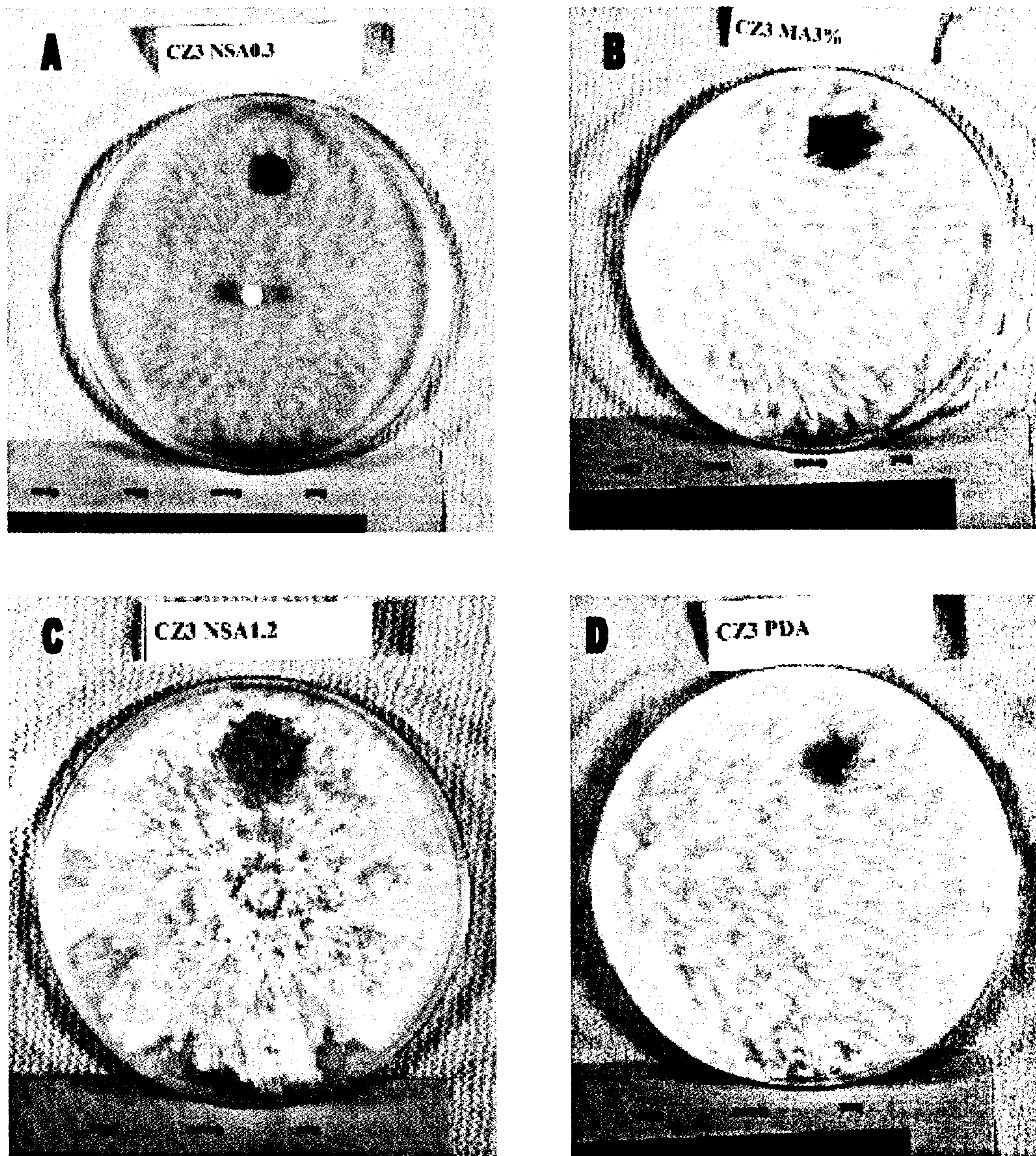

Figure 5.4. The different gum guaiac reactions associated with the growth of *C. subvermispora* on different media after 11 days. (A) shows a strong reaction on NSA0.3, (B) indicates a moderate reaction produced on MA3%, (C) reveals a faint reaction on NSA1.2 and (D) exhibits no reaction on PDA.

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The initial analysis using the Pearson Product-Moment correlation showed positive correlations between all pairs of colour layers: 0.98 between the red and green layers, 0.91 between the green and blue layers, and 0.81 between the red and blue layers. Since all three values are close to +1 the differences among the layers are slight (Schulman 1992). In addition, a scatter plot matrix of all three layers shows the highly correlated nature of the data (see Appendix III, Figure AIII.1). The purpose of analyzing the correlation between the layers was to assess the need to apply a multivariate analysis on the data. Since all three colour layers were so highly correlated I decided to apply a univariate ANOVA to the data. I chose to focus on the blue layer since the enzyme reaction with the gum guaiac produced a blue colour.

The initial analysis of the blue layer showed that the data failed to satisfy the ANOVA assumptions that the residuals be normally distributed with homogenous variances across treatment groups. A variety of transformations were applied to the data yet normality and homogeneity could not be achieved. The data that presented the most problems came from the reactions on the CSY medium at both ages. The main difference between the data was that isolate L-6332 caused the gum guaiac to turn a creamy yellow colour at both ages, while isolate CZ-3 showed no reaction or turned the solution faintly blue in one instance. The inconsistency of this data and the lack of repetitions for the treatment combinations did not provide adequate data for analysis. However, by removing the CSY treatment combinations from the analysis, a normalized and homogenous set of data was obtained. The side-by-side dot plots and normal probability plots are shown in Appendix III (Figure AIII.2, AIII.3) . There were two outliers among the residuals that may have suggested a deviation from homogeneity;

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however, using Bartlett's test at the 0.001 level suggested the data were sufficiently homogeneous (Lorenzen and Anderson 1993).

The ANOVA highlighted age x isolate x media as the highest order significant interaction (see Appendix III, Table AIII.2). A plot of the means of this interaction showing the differences among the media at age 11 and 21 is shown in Figure 5.5. A LSD of 25.4 (pixel value of blue layer) was calculated at $\alpha=0.05$ in order to show significant differences between the interactions. One of the major factors of significance that can be observed in Figure 5.5 is the difference between the 11-and 21-day graphs. The lower the average pixel values the darker the blue for each reaction. Thus, the 11th day is shown to produce average pixel values between 25 and 140. At the age of 21 days the pixel values are much higher ranging between 65 and 135 indicating the older mycelium was producing less extracellular oxidase than at the younger age.

Only four significant differences among the isolates were observed. At 11 days enzyme reactions were extremely different between both isolates when grown on NSA0.6 and CS0.15. Cultures grown after 21 days showed significantly different reactions between the two isolates on the media NSA1.2 and NSA0.15.

Gum guaiac reactions on the different media produced many statistically significant differences; however, the most visually distinctive difference occurs between the NSA media and the other media. At 11 days isolate L-6332 produced the darkest reactions when grown on NSA0.3, NSA0.15 and NSA. All three media are significantly different than all other media, with the exception of NSA and CS0.15 which fall within the LSD. Similarly, at the same age, isolate CZ-3 produced the most intense blue reactions on NSA0.6, NSA 0.3, NSA0.15 and NSA with all four media being significantly different than the rest. After 21 days, reactions produced by isolate L-6332

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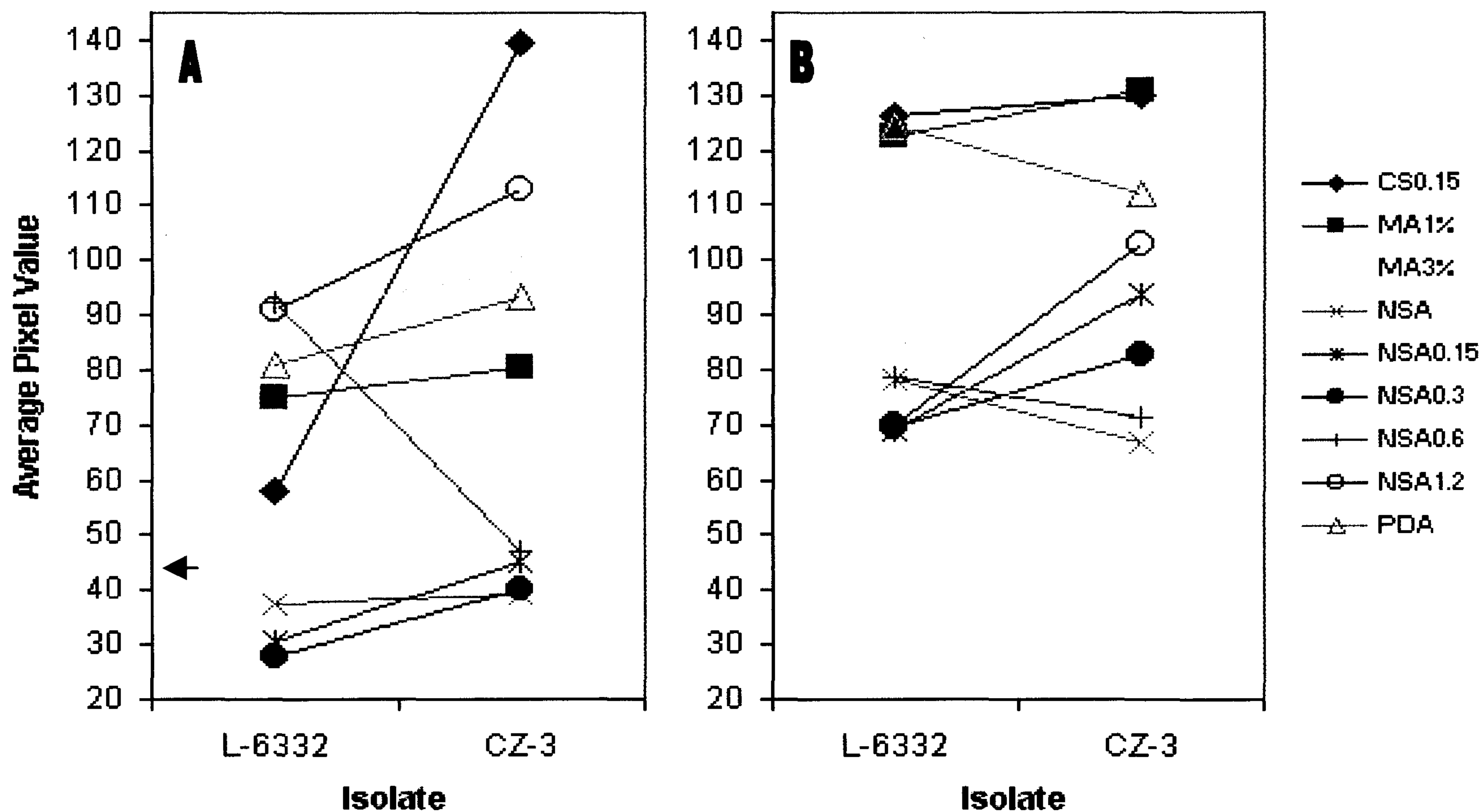

Figure 5.5. Age by isolate by medium interaction plot for the gum guaiac reactions using the blue colour layer (A = 11 days, B = 21 days). The arrow indicates the approximate LSD of 25.4 for both interaction plots. Any group of points that fall within 25.4 pixel values of any others among the day, medium and isolate plots are not significant.

showed an even greater distinction between NSA group (all NSA media) and the non-NSA group (PDA, MA1%, MA3%, and CS0.15). However, reactions with isolate CZ-3 grown after 21 days showed only NSA, NSA 0.3 and NSA0.6 as being significantly different that the non-NSA grouping.

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DISCUSSION

The most significant result produced in this chapter was the difference in extracellular oxidase production between the NSA and non-NSA media. The NSA media was adopted in order to emulate a natural wood medium in a cultural setting. Cultures grown at both 11 and 21 days clearly showed that the NSA media, for the most part, were separated from the rest of the media with the exception of NSA1.2 and 0.6 at 11 days. The darker blue reactions came from the NSA media and were situated down at the lower pixel values. The reaction of both isolates on PDA, MA1% and MA3% were grouped together at higher pixel values for both the 11- and 21-day time periods. CS0.15 was also situated in the higher pixel values for all treatment combinations except for the growth of L-6332 at 11-days. In this instance there was a blue reaction that caused the dramatic difference among the isolates. In all other cases, mycelium on this medium did not produce a blue reaction with the gum guaiac. The glucose levels within the NSA media were observed to have an effect on the polyphenol oxidase reactions in certain treatment combinations. At 11 days the reactions produced by L-6332 were sensitive to the higher glucose levels. Mycelium on NSA1.2 and 0.6 produced virtually no reaction, while the lower glucose levels of 0.15 and 0.3 along with NSA at 0% glucose produced the most significant reactions. A similar situation occurred with isolate CZ-3; however, mycelium grown on NSA0.6 produced a dark reaction and was grouped with NSA0.15, NSA0.3 and NSA. As with L-6332, CZ-3 produced a faint reaction on NSA1.2 indicating that the glucose levels were inhibiting laccase production.

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At 21 days, L-6332 produced strong reactions with all of the NSA media. All five media were not significantly different from one-another. On the other hand, CZ-3 was involved with strong reactions with NSA, NSA0.3 and NSA0.6. Oddly enough mycelium on NSA0.15 had a faint reaction to the gum guaiac; however, as expected NSA1.2 also had a faint reaction.

The reason for the difference in results between the NSA and non-NSA media suggests that the theory of natural linkages (Lobos *et al.* 2001) occurring between substrate and fungi are true. The results clearly indicate that both isolates of *C. subvermispora* are media sensitive. Extractives produced by boiling Norway spruce chips seemed to contain the key nutrients required in the production of extracellular oxidase. Similarly, Niku-Paavola *et al.* (1990) found that media containing pressure ground wood, chemithermochemical pulp or spruce shavings increased laccase and MnP production with *Phlebia radiata.* Analysis of the Norway spruce broth may provide more insight into the required elements for the enhanced expression of ligninolytic enzymes.

The results presented in this chapter provide a possible explanation for cultural studies that have been done to date which failed to produce positive oxidase tests with white-rot fungi. Nobles (1958b) tested the use of gum guaiac on malt agar for a variety of white rot fungi. Although her results were successful for a large portion of the fungi tested, there were a few that did not produce a reaction. Although it is quite conceivable that these fungi do not produce extracellular oxidases it may be possible that these fungi were medium sensitive and would produce positive tests on other media. Similarly Wang and Zabel (1990) used gallic and tannic acids in 1.5% malt extract agar to test for extracellular oxidase reactions. Fungi in the genus *Ceriporiopsis* were found to have a

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negative oxidase reaction on this medium. The results in this chapter indicated that *C. subvermispora* on MA1% was also found to have no reaction. In addition, Lombard and Gilbertson (1965) discovered two white-rot fungi in the genus *Poria* that failed to produce extracellular oxidase with the gum guaiac, tannic and gallic acid. Two other *Poria* species produced a faint positive reaction with the gum guaiac and a negative reaction with the Bavendamm test. Using the Bavendamm method, Davidson *et al.* (1942) found that the white-rot fungus *Stereum frustulosum* produced a consistently negative oxidase reaction when grown on 1.5% malt extract agar. Thus, the potential may exist for error in cases where cultural studies have white-rot fungi labeled as having no extracellular oxidase reactions. A definite solution with *C. subvermispora* was to use a broth from boiled Norway Spruce chips in order to produce a positive reaction. This method for testing the gum guaiac solution should be examined further with the use of other extracellular oxidase testing methods and various broths made from both gymnosperm and angiosperm chips. Furthermore, other white-rot fungi that have tested negative to oxidase in cultural descriptions should be examined using similar methods as described in this chapter. Selection of a broth to use as a growing medium would depend on the natural substrates of the fungus.

Further results presented in this chapter showed that the age of the isolates was a factor in the production of polyphenol oxidase. It seems that less oxidase is produced in the older mycelium. This is most likely why Nobles (1958a) suggested the use of gum guaiac on actively growing cultures. Niku-Paavola *et al.* (1990) found with cultures of *Phlebia radiata* the production of laccase increased early in the first few days of growth and then decreased substantially, as the cultures became older. Stalper's (1978) tested the active margin of growth.

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A surprising result was the difference in the glucose levels for the NSA media. Ruttimann-Johnson *et al.* (1993) and Tapia and Vicuna (1995) found that a low glucose level of 0.1% produced lower levels of extracellular oxidase compared to that of the higher 1% level in liquid cultures. The results in this chapter found the opposite to be true for both isolates growing on NSA media. However, Ruttimann *et al.* (1992b) mentioned that enzyme production was considerably different in liquid cultures compared to the activity of the fungus in a natural environment.

An important observation in the results was the high pixel values of MA3% at both 11 and 21 days. Although both isolates produced a moderate blue reaction on MA3% the treatments are indicated as having higher pixel values than PDA and MA1% at age 11. A reason for this may be from the colour of the gum guaiac solution. When no reaction occurs, the brown colour of the guaiac solution remains unchanged; however, in the case of MA3% the brown colour is changed to a light blue colour. The pixel values for the brown colour may show up darker than the light blue colour in the blue layer, thus it seems as though MA3% is not producing the enzyme. Unfortunately the problem of separating a light blue reaction from a brown reaction may be the drawback of analyzing colour by pixel values.

The pH of the media did not seem to have a major effect on the production of the polyphenol oxidase. Marr (1979) noted that tyrosinase activity occurred in the pH range of 3.5 to 7.1 with on optimum pH of 6 to 7, while laccase activity has a range of 2-3 to 7-9.5 with optimum pH being dependent on the isoenzyme and the substrate. Fukushima and Kirk (1994) indicated the optimal pH for the production of laccase with isolate CZ-3 fell in the range of 3 to 5 and yet the most intensive reactions for extracellular oxidase in my results came from the NSA media which had a pH of 6.5

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(see Table 2.5). This may indicate that the gum guaiac reaction is occurring due to the presence of tyrosinase; however, laccase can also be produced at this pH. It seems that there are other factors, other than pH, to take into consideration when examining the optimal production levels for extracellular enzymes. Marr (1979) states that there are many factors influencing enzyme specificity such as substrate, pH and the type of isoenzyme involved. Thus, examination of specific enzymes using enzyme specific tests could bring about a better understanding of media dependent fungi.

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FINAL CONCLUSIONS FOR THESIS

The cultural characteristics observed in my work were found to be quite consistent with the observations of Domanski (1969), Stalpers (1978) and Nakasone (1981). However, I presented some new observations to the established cultural descriptions. The productive range for the growth of isolate CZ-3 was established to be between 15°C and 35°C with the optimum growth occurring at 30°C. Different types of media were found to influence the macroscopic observations of the hyphae. Aerial hyphae production was highest on PDA and MA3% and was inhibited by NSA0.15. Microscopic analysis of *C. subvermispora* yielded important discoveries. Two different crystal formations were found to be produced by the hyphae in cultures grown on 1% malt extract agar. These crystals may be important secondary metabolites and have commercial potential.

Isolates of *C. subvermispora* were found to be medium dependent, meaning that the type of medium used changed the biochemistry of the fungus. Isolates CZ-3 and L-6332 were specifically examined in this study due to their ability to fruit. The reasons for the failure to initiate fruiting in isolates FP-90031-sp and ME-485 are unknown. Further examination into the initiation of fruiting with these isolates is required. Type of media proved to be a key factor in basidiocarp formation, and MA1% was by far the best medium for producing basidiocarps regularly when the aeration tray method was used. However, it was on the natural wood substrate where fruiting developed the fastest and

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most abundantly. Basidiocarp formation on jack pine boards occurred in one week after exposure to air. Isolate CZ-3 was found to be the highest spore producer and had the highest germination at 72%. The spore production of a small basidiocarp from this isolate was found to be in the billions over a 24-hour period. The potential for basidiospore production from *C. subvermispora,* as a cheap and efficient source of inoculum in biopulping is an important advance in biopulping technology and should be examined in greater detail.

Spore production allowed for examination into the sexuality of the *C. subvermispora*. Nuclear staining of the basidiospores yielded haploid nuclei, thus confirming that the fungus was heterothallic. Staining of the hyphae typically found haploid pairs between septa showing the dikaryotic nature of the fungus. Interfertility tests using single spore isolates clearly demonstrated that this fungus was tetrapolar. My work offers a different perspective to the sexuality of the fungus in addition to the work of Stalpers (1978) and Domanski (1969) who classified *C. subvermispora* as being bipolar.

Many cultural studies have found that some white-rot fungi do not produce extracellular oxidase (Nobles 1958b; Stalpers 1978; Wang and Zabel 1990). A potential explanation for this phenomenon resulted from this study. Commonly used media in cultural studies such as MA1% and PDA were found to produce negative gum guaiac reactions with *C. subvermispora*. However, on a naturally emulated medium consisting of the Norway spruce infusion agar, the gum guaiac reaction was quite strong. Once again the importance of the fungus/media interaction is quite evident. It is obvious that certain nutrients in growing media are utilized by the fungus to produce extracellular enzymes. If these nutrients are not present then the fungus is unable to produce the

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enzymes. Thus, culturally white-rot fungi can be easily mistaken for not having polyphenol oxidases when they acutally do. Furthermore, this finding has implications in the biopulping industry. Treatment of pulp chips with nutrient media such as corn steep liquor (Akhtar *et al.* 1997) should be examined for the effects of the corn steep liquor as an effective initiator of extracellular enzymes. Corn syrup media used in this study failed to produce large amounts of extracellular enzymes. It would be beneficial that if liquid inoculum or pretreatments with liquid media are used, the media should initiate enzyme production in order to maximize the delignification process.

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APPENDIX I

PERSONAL COMMUNICATION, CARDINAL GROWTH DATA, AND KEY CODES

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LAKEHEAD UNIVERSITY
955 Oliver Road, Thunder Bay, Ontario, Canada P7B 5E1
Faculty of Forestry
Telephone (807) 343-8507
Fax (807) 343-8116

Personal Communication

1 October 2002

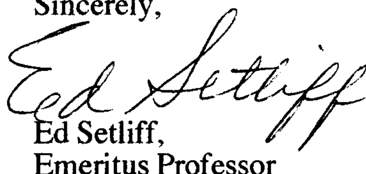
Dear Ryan:

This note is intended to establish some of the things that we have discussed during your thesis research that lie behind the discovery of *Ceriporiopsis subvermispora* as a good biopulping candidate.

My biopulping research was initially supported by a grant from the International Paper Co in the mid-1970s. The grant went to my employer the Cary Arboretum of the N.Y. Botanical Garden in Millbrook, NY. My job was to collect white rot fungi, use them to decay wood, and then send the samples down to Dr. Al Crowder and Dr. Wayne Eudy at IP for lignin analysis. Crowder must have spotted the different rates of reaction to the wood, and we published the results but without the culture names of the fungi (Crowder *et al.* 1978). *Cv* emerged right away as one of the choice isolates for further study as a biopulper. The 1980 paper gave some identity of the cultures, and many of these were obtained from the Center of Forest Mycology Research. The names were understandably not guaranteed for accuracy, and so I largely avoided using names until such time that I might verify their ID.

Many times what underlies advances in science, and whether hastened or delayed, never sees print or is even mentioned to very many people. This is a pity, because we lose so much in terms of the "mechanics" of history from the participants. Here, you have a few lines from my memory.

Sincerely,



Ed Setliff,
Emeritus Professor

ACHIEVEMENT THROUGH EFFORT

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Table AI.1. Growth rate data of *C. subvermispora* after 1- and 2-weeks on 1.5% malt extract agar at 7 temperatures.

Temperature (°C)	Replicate	Growth after 1 week (cm)	Growth after 2 weeks (cm)
10	1	0.0	0.0
	2	0.0	0.0
	3	0.0	0.0
	4	0.0	0.0
15	1	0.0	1.6
	2	0.0	0.0
	3	0.0	2.4
	4	0.0	2.0
20	1	2.2	7.0
	2	2.1	7.0
	3	1.5	6.5
	4	1.5	6.5
25	1	3.3	8.8
	2	3.2	8.8
	3	3.3	8.8
	4	3.2	8.8
30	1	5.0	8.8
	2	5.0	8.8
	3	5.1	8.8
	4	5.2	8.8
35	1	2.0	4.7
	2	2.4	4.7
	3	1.8	4.5
	4	1.9	3.8
40	1	0.0	0.0
	2	0.0	0.0
	3	0.0	0.0
	4	0.0	0.0

Temperature (°C)	Average Growth (cm)	
	Week 1	Week 2
10	0.0	0.0
15	0.0	1.9
20	1.8	6.8
25	3.3	8.8
30	5.1	8.8
35	2.0	4.4
40	0.0	0.0

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Proposed key codes for *C. subvermispora*:

Davidson, Cambell and Vaughn (1942) Key Code

Codes derived by Nakasone (1981)
A, P, F, 1, 2, 10, 16
A, P, F, 1, 2, 10
A, P, F, 1, 2, 10, 14, 16
A, P, F, 1, 2, 10, 14

Proposed code using isolate CZ-3
A, I, 1, 2, 10, 14

Nobles (1948) Key Code using isolate CZ-3

Proposed code using isolate CZ-3
Column 1 = (1,2)
Column 2 = 1
Column 3 = 2*
Column 4 = 3
Column 5 = 7
Column 6 = 1
Column 7 = 2
Column 8 = 2
Column 9 = 1
Column 10 = 1**
Column 11 = 3

Nobles (1965) Key Code

Code derived by Nakasone (1981)
2, 4, (7), (8), 34, 36, 38, 41, (42), 54, 55, 59

Proposed additions using isolate CZ-3
(26), 40, 42, (48), (59), (60)

Stalpers (1978) Key Code

Existing Code
1, 2, 3, (5), (6), (11), 13, 14, (16), 17, 18, 21, (22), 30, (37), (39), 40, (42), 44, 45, (50), 52, 53, 54, 57, (78), (80), 82, 83, 85, 90, (93)

Proposed additions using isolate CZ-3
(19), (48), 89, (94), (95), 97

*results of enzyme test using gum guaiac
**when cultures are not sealed allowing for exposure to air

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APPENDIX II

SINGLE SPORE MATINGS
AND
THE MICROSCOPIC AND MARCROSCOPIC REACTIONS

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Single-Spore Matings	Left Side	Response Zone of Contact	Right Side	Macroscopic Reaction	Frequency of Clamps or Pseudoclamps	Mating Types
MxH	-	+pseudo	-	Barrage	moderate	A1B1xA2B1
ExO	-	+pseudo	-	Barrage	moderate	A1B2xA2B2
IxB	-	+pseudo	-	Flat	moderate	A2B2xA1B2
CxA	-	+pseudo	-	Flat	moderate	A1B1xA2B1
JxG	-	+pseudo	-	Flat	moderate	A1B2xA2B2
OxB	-	+pseudo	-	Flat	moderate	A2B2xA1B2
OxJ	-	+pseudo	-	Barrage	moderate	A2B2xA1B2
HxD	-	+pseudo	-	Barrage	moderate	A2B1xA1B1
KxB	-	+pseudo	-	Barrage	moderate	A2B2xA1B2
HxC	-	+pseudo	-	No reaction	moderate	A2B1xA1B1
IxJ	-	+pseudo	-	Barrage	moderate	A2B2xA1B2
DxA	-	+pseudo	-	Barrage	very few	A1B1xA2B1
ExI	-	+pseudo	-	Flat	moderate	A1B2xA2B2
OxM	-	+	-	Flat	moderate	A2B2xA1B1
MxK	-	+	-	Barrage	moderate	A1B1xA2B2
LxG	-	+pseudo	+pseudo	Barrage	very patchy in both areas	A1B2xA2B2
NxB	-	+pseudo	+pseudo	Barrage	B side very few	A2B2xA1B2
GxB	-	+pseudo	+pseudo	Barrage	moderate	A2B2xA1B2
KxL	-	+pseudo	+pseudo	Barrage	moderate	A2B2xA1B2
NxL	+pseudo	+pseudo	-	Barrage	very few in both areas	A2B2xA1B2
LxO	+pseudo	+pseudo	-	Barrage	very few in both areas	A1B2xA2B2
LxI	+pseudo	+pseudo	-	Barrage	moderate	A1B2xA2B2
NxJ	-	+pseudo	+pseudo	Barrage	moderate	A2B2xA1B2
NxE	-	+pseudo	+pseudo	Barrage	moderate	A2B2xA1B2
NxD	+	+	-	Barrage	few on N side	A2B2xA1B1
OxC	-	+	+	Barrage	high	A2B2xA1B1
HxJ	-	+	+	Barrage	high	A2B1xA1B2
HxE	-	+	+	Barrage	high	A2B1xA1B2
HxL	-	+	+	Barrage	high	A2B1xA1B2
MxA	+pseudo	+pseudo	+pseudo	Barrage	very few in all three areas	A1B1xA2B1
KxE	+pseudo	+pseudo	+pseudo	Barrage	very few in all three areas	A2B2xA1B2
GxE	+pseudo	+pseudo	+pseudo	Barrage	very few in all three areas	A2B2xA1B2
IxC	+	+	+	No Reaction	high, moderate on C side	A2B2xA1B1
KxD	+	+	+	Barrage	high	A2B2xA1B1
AxL	+	+	+	Barrage	high, moderate on A side	A2B1xA1B2
JxA	+	+	+	No Reaction	high	A1B2xA2B1
NxC	+	+	+	No Reaction	high	A2B2xA1B1
GxC	+	+	+	No Reaction	high	A2B2xA1B1
MxG	+	+	+	No Reaction	high, patchy on M side	A1B1xA2B2
BxA	+	+	+	Barrage	high	A1B2xA2B1
KxC	+	+	+	Barrage	high	A2B2xA1B1
ExA	+	+	+	No Reaction	high	A1B2xA2B1
NxM	+	+	+	Barrage	high	A2B2xA1B1
HxB	+	+	+	No Reaction	high	A2B1xA1B2
MxI	+	+	+	No Reaction	high	A1B1xA2B2
OxD	+	+	+	Barrage	high	A2B2xA1B1
IxD	+	+	+	Barrage	high, few on I side	A2B2xA1B1
GxD	+	+	+	Barrage	high, few on G side	A2B2xA1B1

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Single-Spore Matings	Left Side	Response Zone of Contact	Right Side	Macroscopic Reaction	Frequency of Clamps or Pseudoclamps	Mating Types
KxO	-	-	-	Flat	N/A	A2B2xA2B2
NxK	-	-	-	Flat	N/A	A2B2xA2B2
MxC	-	-	-	Flat	N/A	A1B1xA1B1
KxJ	-	-	-	Flat	N/A	A2B2xA1B2
ExJ	-	-	-	No reaction	N/A	A1B2xA1B2
BxE	-	-	-	Flat	N/A	A1B2xA1B2
LxE	-	-	-	No reaction	N/A	A1B2xA1B2
BxJ	-	-	-	Flat	N/A	A1B2xA1B2
LxJ	-	-	-	Flat	N/A	A1B2xA1B2
LxB	-	-	-	Flat	N/A	A1B2xA1B2
OxN	-	-	-	Flat	N/A	A2B2xA2B2
IxN	-	-	-	Flat	N/A	A2B2xA2B2
GxN	-	-	-	No reaction	N/A	A2B2xA2B2
IxK	-	-	-	Flat	N/A	A2B2xA2B2
GxK	-	-	-	Flat	N/A	A2B2xA2B2
IxO	-	-	-	Flat	N/A	A2B2xA2B2
GxO	-	-	-	Flat	N/A	A2B2xA2B2
GxI	-	-	-	Flat	N/A	A2B2xA2B2
DxE	-	-	-	Flat	N/A	A1B1xA1B2
CxE	-	-	-	Flat	N/A	A1B1xA1B2
MxE	-	-	-	Flat	N/A	A1B1xA1B2
JxD	-	-	-	Flat	N/A	A1B2xA1B1
JxC	-	-	-	No reaction	N/A	A1B2xA1B1
JxM	-	-	-	No reaction	N/A	A1B2xA1B1
DxB	-	-	-	Flat	N/A	A1B1xA1B2
CxB	-	-	-	Flat	N/A	A1B1xA1B2
MxB	-	-	-	Flat	N/A	A1B1xA1B2
DxL	-	-	-	Flat	N/A	A1B1xA1B2
CxL	-	-	-	No reaction	N/A	A1B1xA1B2
MxL	-	-	-	Flat	N/A	A1B1xA1B2
HxN	-	-	-	No reaction	N/A	A2B1xA2B2
HxK	-	-	-	No reaction	N/A	A2B1xA2B2
HxO	-	-	-	No reaction	N/A	A2B1xA2B2
HxI	-	-	-	No reaction	N/A	A2B1xA2B2
HxG	-	-	-	No reaction	N/A	A2B1xA2B2
AxN	-	-	-	Flat	N/A	A2B1xA2B2
AxK	-	-	-	Flat	N/A	A2B1xA2B2
AxO	-	-	-	Flat	N/A	A2B1xA2B2
AxI	-	-	-	No reaction	N/A	A2B1xA2B2
AxG	-	-	-	No reaction	N/A	A2B1xA2B2
CxD	-	-	-	Flat	N/A	A1B1xA1B1
MxD	-	-	-	No reaction	N/A	A1B1xA1B1
AxH	-	-	-	Flat	N/A	A2B1xA2B1

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APPENDIX III

AVERAGE PIXEL VALUES AND ANALYSIS FOR THE EXTRACELLULAR OXIDASE REACTIONS

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Table AIII.1. Average pixel values for the extracellular oxidase reactions.

Age	Strain	Medium	Rep	Red Layer	Green Layer	Blue Layer
21	L-6332	NSA1.2	1	139.583	111.9670511	75.2774
21	L-6332	NSA1.2	2	148.5904	110.7580915	64.37914
21	L-6332	NSA0.15	1	92.51683	89.3278384	76.45571
21	L-6332	NSA0.15	2	216.0978	150.0653633	61.51071
21	L-6332	NSA0.3	1	212.6112	149.1820362	74.41114
21	L-6332	NSA0.3	2	177.4715	126.8007394	64.24317
21	L-6332	NSA0.6	1	167.9365	124.1286656	79.94723
21	L-6332	NSA0.6	2	166.8042	130.7758866	76.93925
21	L-6332	NSA	1	139.2735	113.45124	78.03696
21	L-6332	NSA	2	135.3072	111.7404846	78.1343
21	L-6332	CSY	1	225.6997	189.2208437	124.3026
21	L-6332	CSY	2	245.9619	215.5634206	149.0053
21	L-6332	CS0.15	1	216.303	179.3949672	120.4717
21	L-6332	CS0.15	2	228.4132	194.8688427	131.5511
21	L-6332	MA1%	1	231.4547	189.9357483	117.1745
21	L-6332	MA1%	2	229.3739	194.1500827	127.6732
21	L-6332	MA3%	1	155.8958	145.6910776	111.9085
21	L-6332	MA3%	2	157.5267	145.2677761	108.2532
21	L-6332	PDA	1	250.8447	209.0452591	129.1834
21	L-6332	PDA	2	245.9907	199.2565953	119.4766
21	CZ-3	NSA1.2	1	140.9283	122.0122696	90.79681
21	CZ-3	NSA1.2	2	209.5064	171.9590927	114.6238
21	CZ-3	NSA0.15	1	171.1277	144.0325125	95.31957
21	CZ-3	NSA0.15	2	177.5532	146.8649617	92.39242
21	CZ-3	NSA0.3	1	215.8991	178.4555057	119.0602
21	CZ-3	NSA0.3	2	178.6499	114.277796	46.57564
21	CZ-3	NSA0.6	1	124.393	103.5120702	71.21127
21	CZ-3	NSA0.6	2	136.5429	108.0337024	71.26994
21	CZ-3	NSA	1	109.5067	86.54524415	61.35527
21	CZ-3	NSA	2	110.2081	92.19087076	71.999
21	CZ-3	CSY	1	174.3157	138.2427758	86.01174
21	CZ-3	CSY	2	186.5628	190.9866353	159.9424
21	CZ-3	CS0.15	1	227.0601	196.3883623	131.7086
21	CZ-3	CS0.15	2	225.0969	191.1241727	127.67
21	CZ-3	MA1%	1	238.8634	198.6092682	126.4007
21	CZ-3	MA1%	2	244.4605	208.309108	135.1946
21	CZ-3	MA3%	1	253.7418	201.9084716	117.1538
21	CZ-3	MA3%	2	254.2971	204.159744	124.1514
21	CZ-3	PDA	1	237.1442	179.539834	98.08643
21	CZ-3	PDA	2	249.3805	205.6692142	126.188

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Table AII.1. continued...

Age	Strain	Medium	Rep	Red Layer	Green Layer	Blue Layer
11	L-6332	NSA1.2	1	172.7398	146.8744487	93.67488
11	L-6332	NSA1.2	2	202.9587	159.5078545	88.21616
11	L-6332	NSA0.15	1	60.56905	51.25365512	39.35501
11	L-6332	NSA0.15	2	61.47775	45.38333148	21.83621
11	L-6332	NSA0.3	1	51.6247	48.61945339	36.38023
11	L-6332	NSA0.3	2	33.28825	26.20999242	19.48777
11	L-6332	NSA0.6	1	220.1847	172.4479799	94.07577
11	L-6332	NSA0.6	2	223.7906	172.9942561	90.4206
11	L-6332	NSA	1	38.99926	36.87878788	32.93974
11	L-6332	NSA	2	50.25366	47.27112692	41.79078
11	L-6332	CSY	1	228.9166	196.0354466	134.0565
11	L-6332	CSY	2	228.7275	195.8905435	133.9398
11	L-6332	CS0.15	1	77.18092	65.25210194	44.9492
11	L-6332	CS0.15	2	204.3991	153.3427467	71.50824
11	L-6332	MA1%	1	195.1797	158.5194009	89.06411
11	L-6332	MA1%	2	191.1684	133.6800933	61.02579
11	L-6332	MA3%	1	159.6187	140.1412226	88.14441
11	L-6332	MA3%	2	163.3224	141.8095615	91.87468
11	L-6332	PDA	1	220.2829	175.7907304	97.18948
11	L-6332	PDA	2	196.2856	142.0623105	64.26939
11	CZ-3	NSA1.2	1	194.353	167.4605301	111.4983
11	CZ-3	NSA1.2	2	186.265	161.8019428	114.1414
11	CZ-3	NSA0.15	1	47.87822	54.70466907	49.75323
11	CZ-3	NSA0.15	2	45.75523	47.29750849	40.92035
11	CZ-3	NSA0.3	1	38.72948	37.00303636	43.85858
11	CZ-3	NSA0.3	2	64.07943	53.9713954	36.42801
11	CZ-3	NSA0.6	1	95.99767	78.99175505	48.43113
11	CZ-3	NSA0.6	2	63.11674	56.95566148	45.86636
11	CZ-3	NSA	1	41.31258	40.99136424	38.98319
11	CZ-3	NSA	2	55.81196	54.72735839	39.45472
11	CZ-3	CSY	1	202.1863	145.7110996	68.51028
11	CZ-3	CSY	2	202.5438	146.6704262	69.78022
11	CZ-3	CS0.15	1	214.3656	191.3284756	139.2445
11	CZ-3	CS0.15	2	212.2191	189.9172377	139.695
11	CZ-3	MA1%	1	221.7349	169.9396999	82.43023
11	CZ-3	MA1%	2	223.4251	166.5668611	78.14671
11	CZ-3	MA3%	1	166.2677	147.3491064	102.577
11	CZ-3	MA3%	2	160.1008	141.6950105	94.07233
11	CZ-3	PDA	1	216.3806	168.4888279	88.53178
11	CZ-3	PDA	2	229.4177	183.3656249	98.37575

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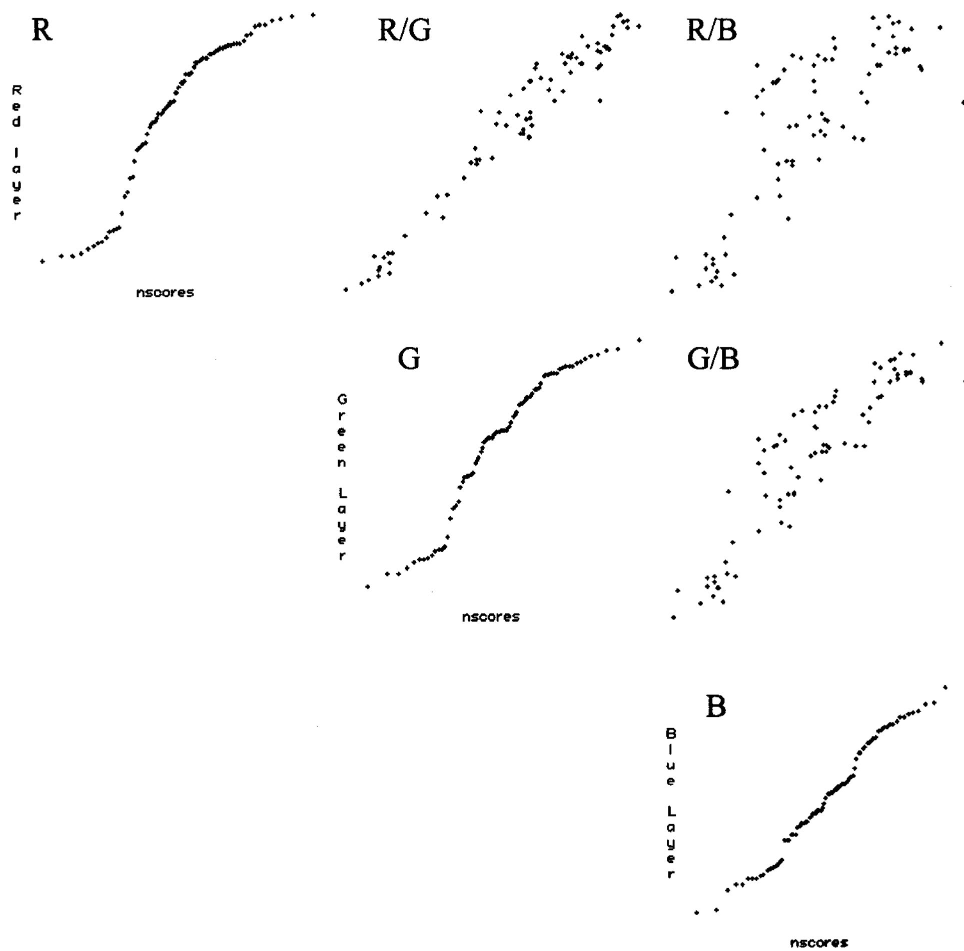

Figure AIII.1. Plot matrix of the average means for the red, green and blue layers. (R) represents the red layer data, (G) represents the green layer data and (B) represents the blue layer data. The data in each layer are compared against one another in plots R/G, R/B and G/B. All three interactions show a high correlation amongst the data.

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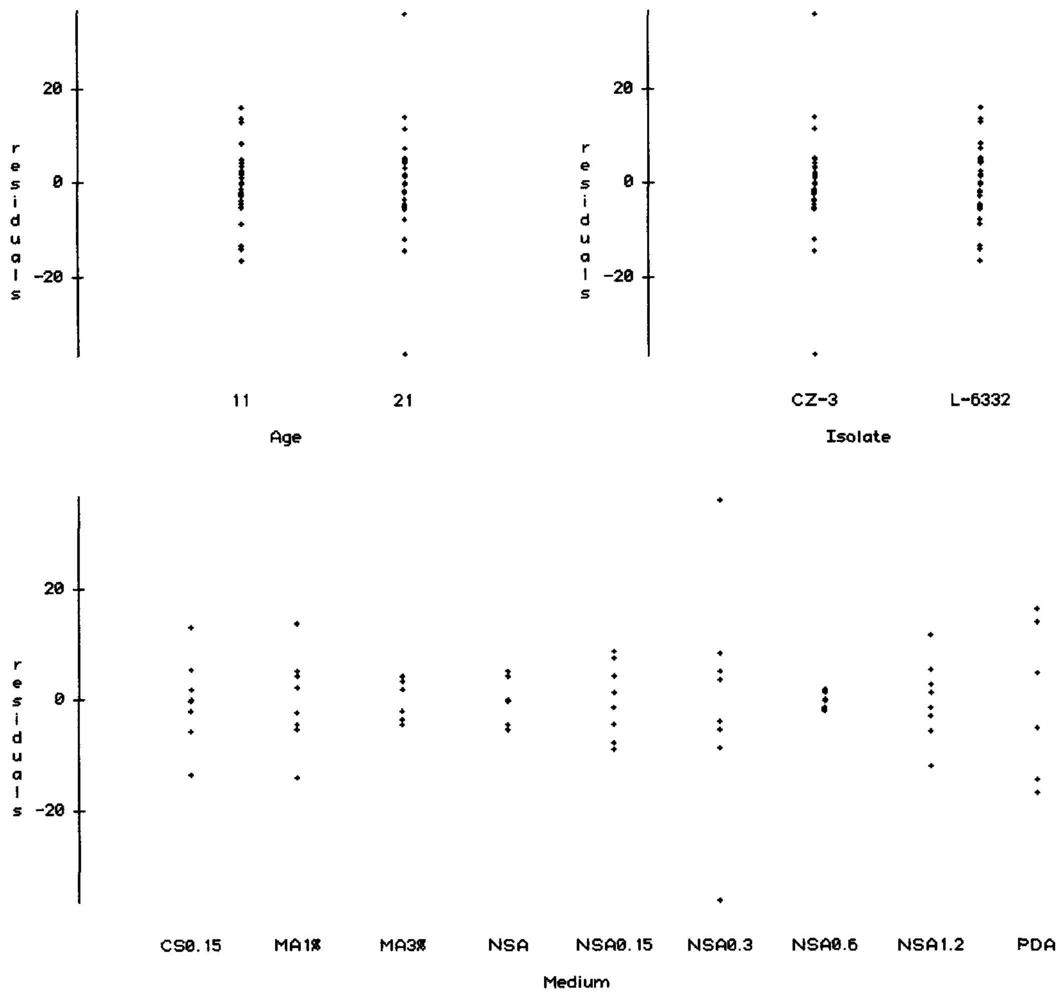

Figure AIII.2. Side-by-side dot plots of the residuals for the oxidase reactions of age, isolate and medium indicating homogeneity across all treatment groups.

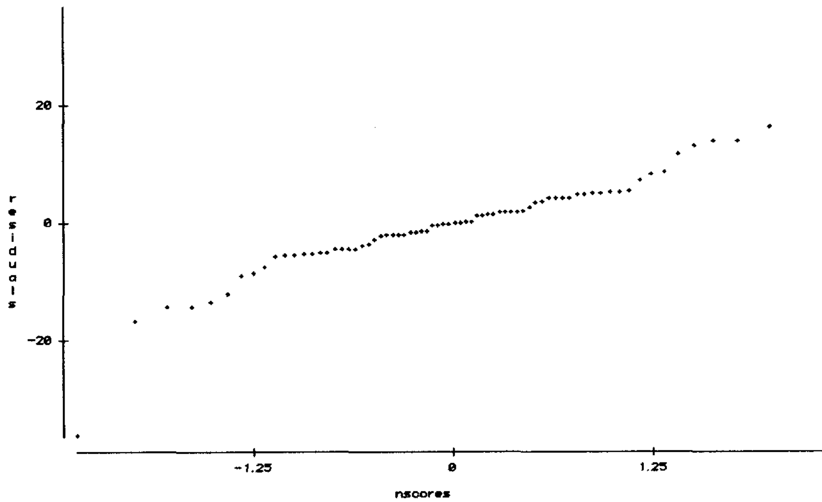

Figure AIII.3. Normal probability plot of the residuals for the blue layer data set showing normality within each treatment.

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Table AIII.2. ANOVA for the blue layer.

Source	df	Sums of Squares	Mean Square	F-ratio	Prob
Const	1	512544	512544	3263.2	^{2}0.0001
Age	1	12735.4	12735.4	81.083	^{2}0.0001
Isl	1	1724.18	1724.18	10.977	0.0021
Age*Isl	1	139.1	139.1	0.88561	0.3529
Mdm	8	35664.4	4458.04	28.383	^{2}0.0001
Age*Mdm	8	6751	843.875	5.3727	0.0002
Isl*Mdm	8	6180.06	772.507	4.9183	0.0004
Age*Isl*Mdm	8	4105.55	513.194	3.2674	0.0067
Error	36	5654.39	157.066		
Total	71	72954			

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