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Biodegradation of canola stubble by *Cyathus olla*

Tracy Charlene Shinners-Carnelley ©

Tracy Charlene Shinners-Carnelley ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Plant Science

Department of Agricultural, Food, and Nutritional Science

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For Mom, Dad, and Trevor

Thank you for your endless love and support

Abstract

This research was conducted to study the decomposition of canola stubble by *Cyathus olla*, and to gather information that may help to develop an innovative strategy for controlling stubble-borne diseases of canola. Accelerated canola stubble decomposition mediated by this fungus may eliminate the overwintering site for pathogens, and thereby reduce disease incidence. The objectives of this thesis were to study the biology, biochemistry, and range of variation in *C. olla,* and to assess the potential of this fungus for development into a microbial inoculant to accelerate canola stubble decomposition.

Scanning electron microscopy, energy dispersive X-ray microanalysis, ^{13}C NMR and FT/ IR spectroscopy, and X-ray diffraction analyses identified calcium oxalate crystals encrusting the hyphae of *C. olla* and *C. striatus* when grown on natural substrata or in culture. Crystal production indicated that these fungi secrete oxalic acid, and sequester calcium from their substrata. This was the first report of oxalic acid secretion by bird's nest fungi.

Cyathus olla basidiocarps were collected from northern and central Alberta, and characterized using 12 morphological parameters and PCR-based RAPD analyses. Much morphological and molecular variability was observed within the 43 accessions characterized, and a new form of *C. olla* was described and named as *C. olla* f. *brodiei.*

Anion exchange chromatography and enzyme assays were carried out to determine the plant cell wall degrading enzymes produced by *C. olla* during solid state fermentation of canola root material. Laccase and manganese peroxidase were detected after both 1-week and 4-week incubations, and aryl-alcohol oxidase was detected after

4-weeks. Polygalacturonase was detected in both incubations, but cellulases were not. This was the first report of manganese peroxidase, laccase, and polygalacturonase production by *C. olla*.

Compositional analysis of canola root material following solid state fermentation with *C. olla* verified that *C. olla* does degrade canola stubble. All forms of *C. olla* significantly reduced the dry weight; and lignin and hemicellulose fractions of canola root material during a 45 day incubation period. *Cyathus olla* f. *brodiei* was the most effective at reducing these fractions.

These results determined that *C. olla* has the biological and biochemical attributes to accelerate canola stubble decomposition, and justifies continued research towards the development of a microbial inoculant.

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List of Abbreviations

AAO	aryl alcohol oxidase
ADF	acid detergent fiber
ASTM	American Society for Testing and Materials
cv	cultivar
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylene diamine tetra acetic acid
FT/ IR	fourier transform infrared spectroscopy
LiP	lignin peroxidase
MnP	manganese peroxidase
NDF	neutral detergent fiber
NDS	neutral detergent soluble
NMR	nuclear magnetic resonance
NTSYS	numerical taxonomy and multivariate analysis system
PCR	polymerase chain reaction
PDA	potato dextrose agar
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
TBE	Tris borate
TE	Tris-EDTA
UAMH	University of Alberta Microfungus Collection
UPGMA	unweighted pair group method of arithmetic averages

Chapter 1

Literature Review

1.1 Introduction

On a global level, the goal of agricultural production is to develop a sustainable system that provides successful management of natural resources and satisfies human needs, while maintaining the environment (FAO, 1989; Conway, 1996). One main objective of sustainable agricultural systems is to reduce inputs into crop production (Conway, 1996). This objective may be achieved by an integrated approach to crop and pest management. Conservation tillage is one such practice that is being widely adopted as part of a sustainable, resource conservation approach to agriculture. This cultural practice has many effects on the agroecosystem, and has brought forth a need for crop residue management with respect to plant disease control.

The retention of crop residues on the soil surface is advantageous in that it decreases soil erosion due to wind and water, improves soil water conservation, and influences soil temperature (Larson *et al.*, 1978; Blevins and Frye, 1993). The movement from conventional to conservation tillage methods influences many physical and chemical properties of soil simultaneously (Blevins and Frye, 1993; Grant and Lafond, 1994; Van Doren and Allmaras, 1978; Prasad and Power, 1991; Schomberg *et al.*, 1994a; Kladivko, 1994), and so alters many facets of the soil microenvironment, including microbial activity (Doran, 1980).

These changes in tillage practices have had varying effects on plant diseases. The retention of crop residues provides a habitat for certain pathogens to overwinter and proliferate. Historically, cultural practices such as tillage and burning were used to eradicate these potential sources of inoculum (Howard, 1996), but these practices are no longer considered sustainable, or environmentally responsible. There are many accounts of changes in plant pathogen populations in response to tillage practices (Bailey and Duczek, 1996; Rothrock, 1992; Sumner *et al.*, 1981; Stover *et al.*, 1996; Bailey *et al.*, 1992; Cook and Haglund, 1991). For these reasons, plant disease control and pest management strategies in general, must be developed to control potentially new pest problems associated

with shifts in methods of crop production. Pest management practices should support a sustainable agricultural system, which poses a great challenge for future research. The development of integrated management strategies requires input of chemical, biological, and cultural control methods and should provide effective, economical, and environmentally safe pest management.

Of these control methods, biological controls are the least developed, but are gaining increased attention due to the potential benefits they may provide. The stubble environment is one area that may be conducive to the growth of beneficial organisms and the development of biological control agents. Undoubtedly, there are many antagonistic interactions between stubble-borne pathogens and soil microbes. However, the ecology and biology of these organisms, and the environment they inhabit must be well understood before further development can occur.

The use of antagonistic microbes in the residue environment is an innovative approach to disease control. Also, use of beneficial microbes to accelerate residue decomposition is a potential biological control tactic. It is the purpose of this review to discuss the effects of conservation tillage and crop residue management on plant disease, methods of control, and the potential of a new control strategy aimed at accelerated stubble decomposition using microbes.

1.2 Conservation Tillage

One advance in the development of sustainable agricultural systems has been the movement toward conservation tillage. Serious problems involving soil erosion and water conservation initiated this change in management practices. Conservation tillage is generally referred to as any tillage and planting system that maintains at least 30% of the soil surface covered by residue after planting (Blevins and Frye, 1993). This definition includes direct seeding, reduced tillage, minimal tillage, and no tillage provided the above condition is met. Conservation tillage has been described by some conservationists as the greatest soil conservation practice to come along in the twentieth century (Blevins and Frye, 1993). Baker *et al.* (1996) have stated that "No technique yet devised by mankind has been anywhere near as effective at halting soil erosion and making food production truly sustainable as no-tillage." As the world population continues to increase, demands for

food and fiber add increasing pressure on the agricultural industry to maintain high yields, while the agricultural land base per capita is decreasing. This often forces the cultivation of steep, fragile soils vulnerable to erosion and degradation. Conservation tillage practices are critical in these situations (Blevins and Frye, 1993; Doran and Linn, 1993).

Benefits of conservation tillage can be summarized as effective soil erosion control, increased use of land too steep for farming by conventional tillage, water conservation, less use of fossil fuels normally associated with land preparation, reduced labor requirements, flexibility of planting and harvesting operations that may facilitate double cropping, and less risk of environmental pollution. Some disadvantages are higher herbicide costs, more difficulty in controlling certain weed infestations, increased potential for insect and disease damage, and increased moisture in fields that are already limited by cool, wet soils in the spring (Blevins and Frye, 1993; Doran and Linn, 1993; Baker *et al.*, 1996).

1.2.1 Soil Properties

Conservation tillage and subsequent retention of surface residues alters both physical and chemical properties of the soil. These factors are influenced by climate and soil types, and therefore only a few generalizations can be made regarding the effects of this practice. Specific information must be regionally determined. Changes that do occur in both physical and chemical soil properties are reflected in the soil/ residue microenvironment.

Physical soil properties influenced by tillage practices include organic matter content, soil water, soil temperature, soil aggregation, porosity, and density (Van Doren and Allmaras, 1978); while chemical properties include soil pH, distribution of nutrients, and organic matter. Detailed descriptions of these factors are given by Blevins and Frye (1993), Van Doren and Allmaras (1978), Prasad and Power (1991), Schomberg *et al.* (1994), and Kladivko (1994). Soil moisture and temperature are two factors that are consistently affected by the retention of surface crop residues, and ultimately affect microbial activity including pathogenic populations.

Tillage practices greatly affect water infiltration, water-holding capacity, and evaporation. Plant residues left on the surface slow the flow of water run-off, and so permit greater infiltration. Residues are also important in slowing the rate of water evaporation

from the soil to the air (Blevins and Frye, 1993; Larson *et al.,* 1978; Van Doren and Allmaras, 1978). Surface residue cover shades the soil from the sun, insulates the soil from heat in the air, and impedes the movement of water vapor from the soil to the air, contributing to the development of high humidity and production of condensation within the residue (Blevins and Frye, 1993). In spring, crop residues reduce soil warming and can decrease seed germination. These effects of conservation tillage on soil moisture have increased crop production potential for rain-fed agriculture in semi-arid and arid regions (Doren, 1980). Moisture content influences soil temperature whereby wetter soils are considered colder soils, and as such is an important negative aspect of conservation tillage (Lafond and Derksen, 1996). This is especially true in areas where wet, cold soils in spring are existing limitations to crop production. Conservation tillage may not be suited to these environments, but the cooling effect of soils by surface residues may be an advantage later in the growing season, or in tropical areas where high temperatures limit plant growth.

1.2.2 Microbial Activity

Soil microbiota (fungi, bacteria, and protozoa) and fauna (nematodes, arthropods, millipedes, and worms) are responsible for soil organic matter transformations and nutrient cycling. These transformations are the foundation for plant decomposition, soil aggregation (Lynch and Bragg, 1985), soil tilth, and nutrient availability (Smith *et al.*, 1993). Soil bacteria and fungi compose the largest biomass in the soil environment and are directly involved in residue decomposition, resulting in mineralization and immobilization of plant nutrients. Protozoa may not be directly involved in decomposition, but play a major role in the ecosystem due to their fungivorous and bacterivorous feeding habits. Because of this, protozoans are involved in mineralization of plant nutrients and regulation of the soil microbial biomass (Hendrix *et al.*, 1986; Cochran *et al.*, 1994). Nematodes, arthropods, millipedes, and worms comprise the micro-, meso-, and macrofauna. These organisms are generally involved in the decomposition process by predation of microorganisms, mineralization and subsequent nutrient cycling, and the physical fragmentation of residues (Hendrix *et al.*, 1986; Cochran *et al.*, 1994). Peak mineralization in soils can be associated with faunal grazing, and a decrease in decomposer populations (Haugen-Kozyra, 1989).

The types of microbes colonizing residue at any one time are dependant on the ability of the organism to utilize the available C substrate (plant residues), environmental conditions, and residue placement (Beare *et al.*, 1993; Cochran *et al.*, 1994). These factors influence decomposition, microbial populations, and succession. Initially, decomposition begins with the degradation of simple sugars followed by more complex carbohydrates, proteins, and structural lignified components. Organisms capable of utilizing these various substrates flourish while they are available, and as the substrate is depleted, colonizing populations are succeeded by microbes capable of using the remaining substrates. Bacteria are dominant in the initial stages of decomposition, but fungi are active in the later stages due to their ability to degrade lignocellulose.

Retention of surface residues appears to favor fungal growth, as fungi are more tolerant of the lower moisture content of residues as compared to bacteria. On the contrary, bacteria are favored by incorporation of residues and a higher soil to residue contact (Cochran *et al.*, 1994). In a study to compare conventional and no-till agroecosystems, Hendrix *et al.* (1986) concluded that no-till management systems increase the importance of fungi relative to bacteria as primary decomposers, and in doing so increases the importance of fungi as a resource base for the detritus food web.

The decomposition process is driven by microbial activity, and changes in microbial soil ecology due to tillage practices can have far-reaching effects on the entire soil ecosystem. Temperature and moisture influence microbial activity and overall soil biomass, so it is not surprising that reduced tillage, or retention of crop residues, significantly affects soil microbial and faunal activities by their influences on these soil factors. In field experiments designed to study the effect of various tillage regimes on dryland corn and subsequent effects on microbial activity, Doran (1980) determined that populations of total organisms, bacteria, actinomycetes, and fungi were 10 to 300% higher on residue-treated plots compared with unmulched treatments. Conclusions from this study indicated that microbial activity was affected by soil water, but was also directly influenced by the presence of the crop residue as a nutrient and energy source.

Follett and Schimel (1989) studied the effects of various tillage practices on microbial biomass dynamics over a period of 16 years. Generally, microbial biomass decreased with increased tillage intensity. Over time, tillage reduced available substrate C,

thereby decreasing the ability of the soil microbial biomass to immobilize and conserve mineral N. Haugen-Kozyra (1989) examined soil biota dynamics under zero and conventional tillage practices and reported that zero tillage systems had greater C concentrations and densities of protozoans, nematodes, and mites in the surface layer. Increased faunal grazing reduces microbial levels, but results in an increase of mineral N release. More recently, Lupwayi *et al.* (1998) studied soil microbial diversity as influenced by tillage and crop rotations. Their results indicated that reductions in substrate richness and evenness due to tillage were responsible for the observed reduction in the diversity of bacteria. However, diversity was higher when legume crops were used in the rotation.

In addition to being directly involved in mineralization and immobilization, soil microorganisms and fauna are important for soil aggregation and structure. Analyses of soil aggregates have shown a relationship between increased soil stability, and colonization of soil by fungi, yeasts, and actinomycetes. This may be attributed to the ability of some organisms to mechanically bind soil particles, or by the ability to produce binding agents (mucilaginous substances) through the decomposition of organic materials (Lynch and Bragg, 1985).

The microbial activity described above concentrates mainly on microbes and fauna involved in decomposition and nutrient cycling. The residue microenvironment is a niche for many diverse organisms, and the ecological interactions that occur among them (Metting, 1993). In addition to organisms with varying degrees of saprophytic ability, there are also many plant pathogens, microbial antagonists, parasites, and hyperparasites in the residue microenvironment. The dynamics of these groups influence microbial populations and the residue microbial community.

1.2.3 Decomposition and Nutrient Cycling

Decomposition is a process by which soil microbes utilize residues for metabolic energy, and in doing so, release nutrients for plant growth. This is a complex process, whereby oxidation of C (in plant residues) provides CO_2, ATP, and C for cell synthesis, and results in the mineralization and immobilization of N, P, and S (Smith *et al.*, 1993). Nutrients are released into the system if they are not required for the microbial biomass (net mineralization), but incorporation of these nutrients into microbial cells results in

immobilization. As previously discussed, microbial activity and soil factors such as moisture and temperature are affected by tillage practices. These variables, combined with the chemical nature of crop residues and soil type form a complex interaction that ultimately affects residue decomposition rate and nutrient cycling.

Decomposition is dependent on water for microbial growth and diffusion of nutrients and by-products formed during the decomposition process (Parr and Papendick, 1978). The amount of moisture may fluctuate within surface residues as compared to incorporated residues, and in extreme cases, the biomass may be killed, or exhibit a slower growth rate. Soil microbes differ in their optimal temperature requirements for growth and activity. However, most soil microorganisms are mesophiles with an optimum temperature range of 20-35 °C. Soil temperatures are variable, with the greatest fluctuations being seen at the surface of the soil or residue (Parr and Papendick, 1978).

Aeration of soils is important for decomposition, as oxygen is required for oxidative assimilation and respiration of the microbial biomass. Anaerobic conditions lead to fermentation and formation of acetic, propionic, and butyric acids. A decrease in pH occurs, and the presence of these organic acids may be phytotoxic for plant growth (Smith *et al.*, 1993). The chemical composition of residues is an important factor for determining decomposition rate. Often, the decomposition process is limited by available N due to microbial demand. Because of this, residues with a high C:N ratio (low N and high lignin content) are decomposed at a slower rate and require additional N that will be immobilized in the soil (Schomberg *et al.*, 1994b).

All of these components of decomposition are influenced in some way by tillage and crop residue management practices. Tillage causes a physical disruption of the soil surface, resulting in a temporary increase in O_2 levels in the soil. As well, mixing and incorporation of residues into the soil surface promotes direct contact between soil particles, residues, and microbes (Smith *et al.*, 1993). These conditions are conducive to microbial activity and rapid decomposition of added residues. Both extremes of residue management have associated consequences. No-till, or lack of soil incorporation may increase the potential for losses from the system by volatilization, but incorporation may increase denitrification and leaching (Power and Legg, 1978).

Smith *et al.* (1993) have detailed many accounts of the reduction in soil organic

matter levels due to tillage practices. In a 16-year study by Follett and Schimel (1989), organic matter levels in the top 5cm of the soil of sod, no-till, stubble mulch, and plow treatments were 53.0, 43.3, 37.3, and 30.3 g kg^{-1}, respectively. Buchanan and King (1993) have also determined that losses of C and P from residues were greater and more rapid when residues were buried as opposed to being retained on the soil surface.

Decomposition of crop residues in a no-tillage system is subjected to varying temperature and moisture, and lacks direct proximity to the microbial population. For these reasons, the rate of decomposition under no-till or conservation tillage is reduced, and nutrient cycling is slower (Smith *et al.* 1993; Schomberg *et al.*, 1994b). Based on a canola residue decomposition study conducted at Beaverlodge, Alberta, Canada, Franzluebbers *et al.* (1996) determined that canola stubble lost 2.5 times more mass when buried compared to surface-placed residues during a 47 day period. After 965 degree days ($^{\circ}C>5$), 57% of the mass of buried residue was lost compared to only a 30% loss of surface-placed residues.

Under conservation tillage, organic matter and relatively immobile plant nutrients like P and K, are concentrated in the upper soil layers (Grant and Lafond, 1994; Blevins and Frye, 1993). Over time, the accumulation of organic matter and organic N at the surface of no-till soils provides a valuable source of N. This is supported by the findings of Grant and Lafond (1994) who determined that the total C and N content in the surface 5 cm of soil was higher under reduced tillage than conventional tillage management. In a long-term study to assess the effects of no-tillage on soil properties, Ismail *et al.* (1994) also determined that soil organic C and N; extractable P; exchangeable Ca, Mg, and K; and pH were all significantly higher in the 0 to 5cm depth of no-till soil compared to conventional tillage. However, below this depth, pH, Ca, Mg, and K were higher with conventional tillage. The concentration of organic matter and immobilization of nutrients in the upper layers of the soil prevent loss of plant nutrient resources, and maintain these nutrients in the root zone. These nutrients may not be available for plant growth if they are tied up in residue, or immobilized in the microbial biomass, but predation of the microbial biomass by soil fauna will result in mineralization of plant nutrients.

Inorganic fertilizers are often required to satisfy plant nutritional requirements, but in excess these amendments may pose potential environmental hazards, and are not

considered to be a sustainable practice. Nutrient cycling and plant nutrition have implications for plant disease, as healthy plants are less susceptible to pathogens compared to those suffering from nutrient deficiencies. Optimizing mineralization of crop residues leading to improved nutrient availability for plant growth is dependent on many inter-related factors. Research in this area is needed to develop sustainable methods of satisfying plant nutritional requirements and maintenance of overall plant health.

1.3 Crop Residue and Plant Disease

Conservation tillage and subsequent surface retention of crop residues can be advantageous for soil and water conservation, soil structure, and soil organic matter. However, this change in residue management has had varying effects on plant disease dynamics, and in many cases, the incidence of certain plant pathogens has increased. Cook *et al.* (1978) have stated that crop residues left in the field after harvest can provide a nutritional substrate and a habitat for pathogens to reproduce, affecting the physical environment of the host and pathogen, and by acting as an organic amendment. Such an addition increases microbial activity and subsequent by-products that may affect pathogens or the susceptibility of host plants.

For the production of many crops, pathogen infested residue is frequently left in the field following harvest. Many pathogens produce sclerotia, chlamydospores, oospores, or other propagules that can successfully overwinter, but others rely largely on residues for carryover and / or production of inoculum for the following season. Some are also good saprophytes, enabling them to colonize their host after the plant is dead. Residue infestation by facultative pathogens, however, is limited by their inability to colonize residues already occupied by other colonists (Cook *et al.*, 1978).

As previously discussed, physical factors such as soil temperature and moisture are affected by the retention of crop residues. This not only affects beneficial organisms, but also plant pathogens. The available substrate, humidity, and lower temperatures provided by the residue environment are conducive to certain pathogens, and can lead to severe diseases problems in the absence of adequate control measures.

The effects of tillage and various residue management practices on plant disease have been documented widely in the literature (Bockus and Shroyer, 1998; Rothrock, 1992;

Bailey, 1996; Conway, 1996; Sumner *et al.*, 1981). There are many accounts of tillage practices increasing, decreasing, or having no effects on plant diseases. Sumner *et al.*, (1981) stated that it is difficult to generalize with respect to the influences of tillage on disease, since these entities are strongly controlled by climate and cropping sequence. Bailey *et al.* (1992) expressed the same difficulty in regard to making generalizations about tillage and impact on disease incidence, since disease development is a complex process involving interactions between tillage practices, crop rotations, and specific disease and crop combinations. Regardless of this, the literature contains many reports of plant disease incidence under conservation tillage systems.

Tan spot of wheat, caused by *Pyrenophora tritici-repentis,* is more common in fields where straw remains on the soil surface. Wheat production under reduced or no-tillage systems has resulted in a dramatic increase of tan spot in the Great Plains, USA (Conway, 1996). Bockus and Claassen (1992) assessed the impact of three tillage methods on tan spot under crop rotation and continuous wheat production. Their results determined that plowing significantly reduced tan spot, but under certain conditions, a one-year rotation out of wheat was as effective. In addition to tan spot, some other foliar diseases of wheat influenced by retaining infested residue include septoria nodorum blotch (caused by *Phaeosphaeria nodorum*), septoria avenae blotch (caused by *Phaeosphaeria avenaria*), septoria tritici blotch or speckled blotch (caused by *Mycosphaerella graminicola*), and head blight or scab (caused by *Gibberella zeae*). Stover *et al.* (1996) determined that any retention of residue in a hard red spring wheat monoculture increased early season severity of these diseases, however there were no consistent season long differences observed.

Miller *et al.* (1998) studied the effect of tillage on fusarium headblight of wheat (*Fusarium graminearum*). Results were inconclusive, but suggested that zero tillage resulted in increased seed infection. Inoculation with molecularly characterized strains of *F. graminearum* enabled Miller *et al.* (1998) to monitor *Fusarium* strains that caused infection. In the year following inoculation, the characterized strains accounted for 20% of seed infection from tilled plots, compared to 79% of strains from no-tilled plots. These results confirm that crop residue is an important source of inoculum for this disease.

Nazareno *et al.* (1993) studied the effects of corn residue on the epidemiology of gray leaf spot of corn (caused by *Cercospora zeae-maydis*) in Ohio, USA. This research

concluded that any tillage method that left residue on the surface favored disease development, given favorable environmental conditions. Disease increased with the amount of residue. Incidence of ear rot of corn caused by *Stenocarpella maydis* (Flett and Mc Laren, 1998) and anthracnose of dry bean caused by *Colletotrichum lindemuthianum* (Ntahimpera *et al.*, 1997) have also been shown to be positively correlated with debris left on the surface.

In an attempt to optimize management strategies for gray leaf spot of corn in South Africa, Ward *et al.* (1997) determined that the onset of disease was 23 days earlier under no-till compared to conventional tillage. However, the benefits of soil moisture conservation with the no-till system compensated for the damage caused by disease. Corn yields under conventional tillage were 28 and 209 kg ha^{-1} lower than in stubble treatments leaving 82 and 26% stubble, respectively (Ward *et al.*, 1997).

Residue retention generally increases incidence of a number of foliar diseases, but no-tillage management has been reported to reduce common root rot of wheat (*Bipolaris sorokiniana*) (Mathieson *et al.*, 1990; Rothrock, 1992; Bailey *et al.*, 1992) and take-all of wheat (*Gaumannomyces graminis*) (Bailey *et al.*, 1992). However, take-all of wheat has also been reported to increase as a result of conservation tillage (Cook, 1982).

1.3.1 Stubble-Borne Diseases of Canola

In western Canada, canola (*Brassica napus* and *B. rapa*) has become a major crop. The residue remaining following harvest consists of the upper portion of the plant, which is easily decomposed, and the lower stem and root which in contrast is fairly woody, and resistant to decomposition. In the semi-arid environments in Canada, canola residue may be retained for 3-5 years, or longer under reduced tillage management (Petrie, 1995). The resistant nature of this stubble plays a significant role in the epidemiology of stubble-borne diseases of canola such as blackleg (Fig. 1-1a) and blackspot (Fig. 1-1b).

1.3.1.1 Blackleg

Blackleg of canola/ rapeseed caused by *Leptosphaeria maculans* (anamorph *Phoma lingam*) poses a serious threat to canola production in many parts of the world.

Historical records describe serious losses and epidemics in cruciferous crops caused by this pathogen. A weakly virulent strain of the blackleg pathogen was periodically found in western Canada after 1961. In 1975, the virulent strain was first discovered on rapeseed stubble in east-central Saskatchewan (Gugel and Petrie, 1992). By 1981, the disease incidence increased tenfold, spreading to western areas of the province, and as far east as the Manitoba border. Virulent blackleg reached Alberta by 1983 and Manitoba by 1984, and shortly after winter rapeseed production increased in Ontario, blackleg was considered a significant disease problem in that province (Gugel and Petrie, 1992).

1.3.1.1.1 Occurrence

Blackleg has the potential of becoming a limiting factor in many rapeseed and canola growing areas. The disease becomes common following the introduction of a virulent strain of the pathogen to an area of intense canola/ rapeseed production, often where highly susceptible cultivars are grown (Gugel and Petrie, 1992). In the years following the introduction of the pathogen and subsequent low levels of infection, infested debris accumulates in canola fields, accompanied by an increase in inoculum. Under these circumstances, the disease can reach epiphytotic proportions if the cultivars grown are susceptible and the weather conducive.

Presently, the disease is prevalent in east, central, and southern Alberta and in the Peace Region of the province. The disease remains uncommon in the west central areas of Alberta. In Saskatchewan, the disease has been slow spreading towards the north compared to progression towards the south. Moist conditions in the north permit more field cultivation and hence crop debris is buried and decomposition of infected residues occurs (Petrie, 1995). During the 1998 season in Manitoba, blackleg basal stem cankers were found in 77% of all crops surveyed and the overall mean provincial incidence of blackleg was 11%. This was responsible for a province wide yield loss of 8% (Mc Laren and Platford, 1999).

1.3.1.1.2 Signs and Symptoms

The disease causes symptoms such as damping off of seedlings, leaf spots, and stem and crown cankers. The cotyledons, hypocotyls, leaves, stems, and pods of canola

are attacked by the fungus. Early infections may result in seedling death shortly before or after emergence, and later infections of leaves and cotyledons develop irregular, water soaked, and whitish gray lesions. Numerous black pycnidia appear in the lesions and exude a pink spore mass under favorable moisture and temperature conditions. Stem lesions are similar, but are sunken, eventually crack, and have purple to black margins (Fig. 1-1a). Under severe conditions, lesions at the basal part of the stem will coalesce, forming a dry rot or canker that completely girdles the stem. Plants in this condition lodge and suffer from much restricted moisture and nutrient flow causing premature ripening, shrivelled seed, and pod shattering (Gabrielson, 1983; Martens *et al.*, 1988; Gugel and Petrie, 1992). Infected stems may not exhibit these external symptoms, but internal tissue may be blackened (Kharbanda, 1993). Pod infections cause premature splitting which results in unequal drying of infected and non-infected portions of the pod. Seeds beneath the lesions are shrunken, unsound, pale gray, and pycnidia may be apparent on them (Kharbanda, 1993).

The weakly virulent strain generally attacks later in the season when plants are maturing. The lesions are smaller, shallow, do not crack, and lack the distinct black margin. Infection by this strain causes little damage and therefore is not as economically important as infection by the virulent strain.

1.3.1.1.3 Disease Cycle

Initial dispersal of the pathogen may occur through infected seed, colonized crop residue, or infected weed hosts (Hall, 1992; Williams, 1992). The introduction of disease to a new area (long distance dispersal) occurs by means of seed-borne inoculum as approximately 2% of seed carries infection (Martens *et al.*, 1988). Contaminated seed is not considered to be an important source of inoculum where the fungus is indigenous. Pseudothecia, ascospores, pycnidia, pycnidiospores, and mycelium can be present on colonized crop residues and give rise to infection, however ascospores are considered to be the most important form of primary inoculum and are responsible for localized spread of the disease (Hall, 1992; Petrie, 1993).

Under suitable environmental conditions of temperature, light, and moisture, pycnidia and pseudothecia produce pycnidiospores and ascospores, respectively. These

spores are dispersed by means of rain splash or wind to a susceptible host where they germinate and enter the host through wounds or stomates. Following a symptomless, biotrophic growth period, lesions or cankers develop. Pycnidia found within the lesions exude pink spore masses, resulting in localized infection (Williams, 1992). Canola seedlings germinated from infected seed may emerge with lesions on the leaves or stems (Evans *et al.*, 1995). Infected plant debris accumulates on the soil surface where it becomes a substrate for the pathogen. If two different strains are present on the stubble, they hybridize to produce pseudothecia (sexual fruiting bodies) (Evans *et al.*, 1995). This stubble containing pycnidia, pseudothecia, and mycelium is an overwintering site for the pathogen, and in the following seasons, spores liberated from this substrate perpetuates the disease cycle (Fig. 1-2).

1.3.1.1.4 Inoculum

The pathogen produces ascospores, pycnidiospores, and mycelium that all function as primary sources of inoculum. Pycnidiospores produced on infested plants can incite infection in the same season, but are not regarded as significant since secondary cycles of infection generally do not affect yield (Hall, 1992). Ascospores are the most important source of inoculum for *L. maculans* (Petrie, 1993). Ascocarps, more specifically pseudothecia, develop on infected stubble from the previous year's crop. Under suitable conditions, ascospores are released into the air from infested stubble. Petrie (1995) determined that one year-old stubble infested with *L. maculans* can produce 4000 ascospores/ 10g stubble/ 1.5 hr. Once liberated, spores are disseminated by wind and may travel several kilometers to susceptible crops (Hall, 1992).

The numbers of spores released are directly related to the severity of the disease in the crop that produced the stubble (Hall 1992). Generally, ascospore production is maximized during the second year after crop growth, and declines thereafter (Petrie, 1995). The woody taproot and lower stem of canola are quite lignified, and resistant to degradation. In a semi-arid environment in Saskatchewan, Canada, residue may persist in the field for 3 to 5 years (Petrie, 1995), and the pathogen may continue to sporulate throughout this time (Hall, 1992). Dry surface soil conditions tend to conserve blackleg inoculum. Flooding or burial of infected residues interrupts spore production, which may

continue when optimum conditions are restored. For these reasons, a 4-year rotation out of blackleg susceptible crops is currently recommended in Canada. Hershman and Perkins (1995) studied ascospore discharge from canola stubble in Kentucky. A significant amount of ascospores were released in the fall and winter following harvest, but release was minimal from 10 month-old stubble. However, Hershman and Perkins (1995) suggest that due to the field conditions in Kentucky, decomposition occurs quickly and little stubble remains in the field 1-year post-harvest.

1.3.1.1.5 Control

Current control methods for blackleg include chemical and cultural practices such as crop rotations, and the use of resistant varieties (Stringam *et al.,* 1995). Presently in western Canada, a 4-year rotation is recommended for canola, the time required to decompose a significant amount of infected stubble. However, in view of higher economic returns for this crop, farmers would like to grow canola in shorter rotations.

1.3.1.2 Alternaria Blackspot

Alternaria blackspot, caused by *Alternaria brassicae,* is also a stubble-borne disease of canola that is present on the crop each year, but usually at very low levels (Martens *et al.* 1994). Stubble-borne spores and hyphae of *A. brassicae* become an important source of primary inoculum when canola is continuously cropped (Verma and Saharan, 1994; Kharbanda and Tewari, 1996). The spores are easily spread by wind and rain to susceptible hosts, and ensure perpetuation of the disease.

The disease affects leaves, stems, and pods causing blackspot symptoms. Leaf lesions can be variable depending on environmental conditions. Lesions start as small brown to black spots that enlarge, and may become gray-black in color. The necrotic spots are solid, or develop as concentric circles, either of which may be surrounded by a chlorotic halo (Verma and Saharan, 1994). The lesions found on siliques and stems are small black spots (Fig. 1-1b). Infection of these plant parts is common when the weather is conducive. Photosynthate production by siliques contributes significantly to seed development (Allen *et al.,* 1971), and severe silique spotting can result in reduced yields. Infected siliques may also result in premature ripening, shattering, and seed infection (Tewari and Mithen, 1999).

1.4 Control Tactics for Stubble-Borne Diseases

Stubble-borne or residue-infesting pathogens have been controlled by tillage systems that bury infested residues. This sanitation method prevents above ground pathogens from sporulating and increases decomposition rate of the stubble as compared to decomposition on the soil surface. Although some disease problems have increased as a response to conservation tillage, this negative aspect does not outweigh the benefits for soil and water conservation. In order to control these problems, plant pathologists are faced with developing new strategies that are effective and sustainable. An integrated approach to disease management is perhaps best suited to a sustainable agricultural system. As stated by Bailey (1996) "there will be no single remedy for diseases in reduced tillage systems, control will come from a holistic approach to crop systems and plant health management." Cultural, chemical, and biological controls are of importance to this approach, with biologicals having great potential for future development.

Bailey and Duczek (1996) have suggested optimizing plant growth as an approach to disease management. Proper fertilizer applications, seed placement, weed control, and crop rotation can all promote healthy plant growth and reduce the risk of loss from disease. Fungicide applications may be effective, but often are not economically feasible. In years of disease pressure, crops under no-till management may require chemical control to maintain yields. Cook *et al.* (1978) also discuss the importance of disease resistant cultivars, crop rotation, and possible chemical controls for managing stubble-borne diseases under conservation tillage systems.

Lafond and Derksen (1996) proposed new control strategies aimed at residue management. Increased residue decomposition rate by increasing heat units, N content of residue, or microbial activity may be effective. From a plant breeding perspective, Lafond and Derksen (1996) have suggested that allelopathic properties be bred into crops so that residues can control weed and disease problems. Breeding black pigmentation into straw may serve to increase light absorption, increase the temperature, and therefore increase decomposition. Increased temperature may also help in speedy seed germination in cool soils during spring. These are innovative approaches for controlling pest problems associated with residue management, but these technologies must be thoroughly examined

from environmental and cropping system points of view.

Bailey and Duczek (1996), Cook *et al.* (1978), Conway (1996), and Pfender *et al.* (1993) have all expressed interest in the development of biological controls for stubble-borne pathogens. The residue microenvironment is a dynamic system with many antagonistic interactions occurring naturally. Closer examination of this environment may reveal ecological information necessary to develop biological control strategies. When commenting on residue colonization by pathogens, Cook *et al.* (1978) stated, "we must learn to upset the mechanisms by which pioneer residue colonists keep possession of the residue they occupy. In doing so, we may learn how to replace pathogens with non-pathogens in residues and so achieve biological control."

Biological control of pathogens in the residue environment is a relatively new approach to control. Biocontrol methods have been explored in the soil environment, but this differs significantly from the residue microenvironment. Variable physical factors ultimately affect residue microbes and often present unique ecological problems for antagonists (Pfender *et al.*, 1993). This point stresses the need for a complete understanding of the microbial and environmental interactions of the residue microenvironment. The development of biological control agents for stubble-borne diseases relies on broadening this information base.

Ongoing research has identified potential biological control agents. Pfender *et al.* (1993) determined that inoculum production by *Pyrenophora tritici-repentis* on wheat straw under conservation tillage could be reduced with inoculation of *Limonomyces roseipellis*. This fungus reduced residue-borne primary inoculum in 3 years of a 4-year study. Inoculation of wheat and black oat straw with *Trichoderma harzianum* was successful in reducing the incidence of *Cochliobolus sativus* on wheat, *Fusarium graminearum*, and other *Fusarium* spp. (Fernandez, 1992b). *Trichoderma harzianum* was also effective at decreasing the incidence of *F. graminearum*, *Macrophomina phaseolina*, and *Glomerella glycines* infesting soybean residues (Fernandez, 1992a). These results demonstrate that *T. harzianum* may be an effective straw-inhabiting antagonist for many pathogens, but much ecological and environmental data must be obtained to support these findings.

1.4.1 Innovative Approach to Disease Control

One approach to biological control involves the destruction of the pathogens' food source and habitat. This was accomplished with tillage, but now alternative methods to achieve this must be developed. Accelerated microbial decomposition of crop residues is a potential mechanism of biological control aimed at reducing stubble-borne pathogen populations. This is the foundation for an innovative approach for controlling stubble-borne diseases of canola.

Observations of a bird's nest fungus, *Cyathus olla*, growing and fruiting on canola stubble (Tewari and Briggs, 1995) (Fig. 1-3) has initiated research on the potential of this fungus to degrade canola stubble, and hence reduce the incidence of stubble-borne diseases such as blackleg and blackspot. In the field, colonized stubble had substantial hyphal growth with rhizomorphs emanating from it and growing into the soil (Fig. 1-4). The residues were soft and macerated, indicative of enzymatic activity (Tewari and Briggs, 1995). Blenis *et al.* (1998) have also identified *C. olla* as a potential biological control agent for these diseases. This fungus has been observed in the same agricultural fields from year to year, indicating the ability to withstand intensive agricultural practices. *Cyathus olla* is widespread throughout the temperate climatic zones (Brodie, 1975), and in a two year study to evaluate the occurrence of fleshy fungi in agricultural fields in Tifton, Georgia, USA, *C. olla* was found to be one of the most common fungal species (Baird *et al.*, 1993). *Cyathus olla* is a saprophyte, and there are no reports of this species being pathogenic. However, *C. stercoreus* has recently been associated with fairy rings in turf plots in Minnesota, USA (Mercier *et al.*, 1999).

1.5 The Bird's Nest Fungi

Cyathus spp. are white, wood-rotting fungi commonly found colonizing plant residues around the world (Brodie, 1975). The common name is descriptive of the unique basidiocarp type characteristic to all members of the Nidulariaceae. In 1975, Dr. H. Brodie recognized forty-two species of *Cyathus*, the most common being *C. olla*. This bird's nest fungus is the most abundant species in Europe and North America and collections have been made north of Sweden and southern South America, however there are no records of *C. olla* from warm moist tropical areas (Brodie, 1975). Variable characters

such as size, color and form are observed within this species, which has led many mycologists to believe that they have discovered a new species.

1.5.1 Classification

This species of bird's nest fungus is classified as:

Division: Eumycota

Sub-division: Basidiomycotina

Class: Gasteromycetes

Order: Nidulariales

Family: Nidulariaceae

Genera: *Cyathus*

Species: *Cyathus olla*

Formae: *C. olla* f. *olla* (Brodie, 1952), *C. olla* f. *anglicus* (Brodie, 1952), *C. olla* f. *lanatus* (Brodie, 1978)

1.5.2 Basidiocarps

The identification and classification of this family of fungi is based on basidiocarp (fruiting body) morphology which is very distinct, and representative of the common name, bird's nest fungus. The basidiocarp is a cup-like structure, consisting of a peridium which is differentiated into three distinct layers and forms the outer boundary wall, giving the fruiting body a relatively rigid structure. The histology of the peridium differs among genera.

Inside the peridium are approximately 15-20 egg-like structures known as the peridioles. The peridiole wall is firm, and often surrounded by a delicate outer covering known as the tunica. At maturity, the central portion of the peridiole is composed of a powdery mass of basidiospores, free from basidia and often intermingled with hyphal fragments. Each peridiole is attached to the base of the peridium by a funiculus which is composed of a sheath, middle piece, and purse. Upon dispersal, the funiculus detaches and the peridiole is ejected from the basidiocarp (Brodie, 1975).

Cyathus olla basidiocarps are typically gray-white to fawn in color and approximately 10-15 mm high and 8-10 mm in diameter. The peridia are expanded or

flared near the mouth and mostly thick walled. The outer surface is fine-textured, with the inner surface being smooth, and frequently transversely ridged. Peridioles are large, and irregular in shape (Brodie, 1975).

1.5.3 Raindrop Dispersal Mechanism

The dispersal mechanism of this group of fungi is unique and exemplifies the great diversity and specialization found throughout the fungal kingdom. "The Nidulariaceae probably possess the most complex spore dispersal mechanism of any of the fungi" (Flegler and Hooper, 1978). The raindrop dispersal mechanism is triggered as raindrops fall into a water filled fruiting body (Fig. 1-5 a & b). The displacement of water within the cup results in an upward and outward thrust that detaches the funiculus, and initiates a series of events (Fig. 1-5 c). The peridiole is ejected and the force on the funiculus causes the purse to open near the attachment to the middle piece, exposing the funicular cord. The coiled hyphae attached to the end of the funicular cord quickly expand and forms the adhesive hapteron. As the peridiole is in flight, the hapteron may adhere to vegetation, causing the funicular cord to pull from the upper part of the purse (Fig. 1-5 d). The cord is extremely elastic and may extend for three inches or more. Once the cord is fully extended, the peridiole is jerked and becomes entangled with the point of attachment (Fig. 1-5 e & f). The adhesive nature of the hapteron ensures the placement of the peridiole, where it may break and disperse spores, or remain intact and be eaten and dispersed by animals (Brodie, 1951).

1.5.4 Biochemistry

Cyathus spp. are biochemically active, producing many enzymes (Kuhad and Johri, 1991), and an antibiotic complex specific to the genus (Allbutt *et al.*, 1970). *Cyathus* spp. are known to produce manganese peroxidase (Orth *et al.*, 1993) cellulases, laccase, phenoloxidase, and xylanase when grown on lignocellulosic materials (Kuhad and Johri, 1991), and aryl-alcohol oxidase activity was recently reported for the first time in *C. olla* (Pelaez *et al.*, 1995). Wicklow *et al.* (1980) were the first to examine and quantify the ability of a bird's nest fungus to degrade lignin. Their results indicated that *C. stercoreus* reduced lignin content of wheat straw by 45% during a 62-day fermentation at 25°C.

Because of this capacity to degrade lignin, *Cyathus* spp. have been evaluated in many applications to delignify and reduce the dry weight of plant residues. *Cyathus* sp., *C. helenae,* and *C. striatus* were all effective in reducing both percent lignin and dry matter of sugarcane baggase (Kuhad and Johari, 1987), and *C. helenae* reduced the dry weight of rice husk by 13 to 36% (Kuhad and Johri, 1991). *Cyathus stercoreus* has been evaluated extensively for its potential to delignify and improve the digestibility of rice straw (Karunanandaa *et al.,* 1995; Karunanandaa *et al.,* 1992); maize stover (Chen *et al.,*1995; Karunanandaa *et al.,* 1992), wheat straw (Agosin *et al.,* 1985), and bermuda grass stems (Akin *et al.,* 1995). Preferential degradation of hemicellulose was observed (Karunanandaa *et al.,* 1992; Chen *et al.,*1995) and enhanced digestibility was obtained in all experiments with the exception of that of Karunanandaa *et al.* (1995).

Cyathus spp. are specialized in residue colonization and biodegradation, however they do appear to have a substrate preference and vary in their ability to degrade different residues (Abbott and Wicklow, 1984). Wicklow *et al.* (1984) studied the ability of 12 different species of *Cyathus,* and two additional taxa in the Nidulariaceae, to degrade wheat straw and silver maple hardwood. Results indicated that each species degraded wheat and hardwood lignin differently, and the ecological specialization of the fungus was not indicative of its ability to degrade the different substrates. In a preliminary study, *C. olla* colonized canola, wheat, and barley residue, but appeared to have a preference for the woody taproots of canola as compared to the cereal residue (Tewari and Briggs, 1995).

In addition to these agricultural systems, there are a few reports in the literature of *Cyathus* spp. being successfully used to degrade other aromatic ring compounds like triphenylmethane dyes (Vasdev *et al.,* 1995) and the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) (Bayman *et al.,* 1995).

Metabolites isolated from *C. helenae* have been characterized and determined to have antimicrobial activity. The complex of antibiotic substances is collectively referred to as cyathin, and has inhibitory effects on actinomycetes, fungi, and bacteria. Some of the most sensitive fungi include *Aspergillus, Penicillium, Fusarium, Trichoderma, Chaetomium,* and *Gliocladium* (Allbutt *et al.,* 1970).

These ecological and biochemical attributes make *Cyathus* spp. excellent candidates as microbial inoculants to accelerate crop stubble decomposition in the field and

hence reduce pathogen populations. The purpose of this investigation is to focus specifically on *C. olla* and its ability to accelerate canola stubble decomposition. Frequent observations of *C. olla* growing and fruiting on canola stubble suggests that this fungus has the capability to proliferate in the field under environmental conditions suited to canola production, and to withstand common management practices such as tillage and herbicide use. In order to pursue the usefulness of this fungus for the proposed application, much information is needed pertaining to the biology, ecology, and biochemical activity of *C. olla* in the stubble environment, and interactions with pathogens, other microbes, and crop plants. It is anticipated that increased population levels of *C. olla* will lead to colonization and accelerated decomposition of canola residues. Elimination of the food source and habitat of pathogens would reduce inoculum levels, and hence the incidence of disease. In addition to disease management, accelerated stubble decomposition may also contribute to nutrient cycling through mineralization of essential plant nutrients tied up in the surface residues.

1.6 Objectives

The objectives were to study the biology, biochemistry, and range of variation within *Cyathus olla,* and to assess the potential of this fungus for development into a microbial inoculant intended to accelerate canola stubble decomposition. The specific objectives are outlined as follows:

1. To examine field-colonized canola stubble using scanning electron microscopy and X-ray microanalysis.

2. To collect *C. olla* from canola stubble in Alberta, and characterize the isolates based on morphological parameters and PCR-based RAPD markers.

3. To detect and identify plant cell wall degrading enzymes produced by *C. olla* when grown on canola stubble.

4. To determine the structural composition of the basal stem and root of canola, and quantify changes in cell wall components of this plant material following incubation with *C. olla.*

1.7 Figures

Figure 1-1. Blackleg and blackspot disease of canola.

(a) Blackleg stem canker caused by *Leptosphaeria maculans*.

(b) Alternaria blackspot stem and silique lesions caused by *Alternaria brassicae*. Photo courtesy of Dr. I. Evans

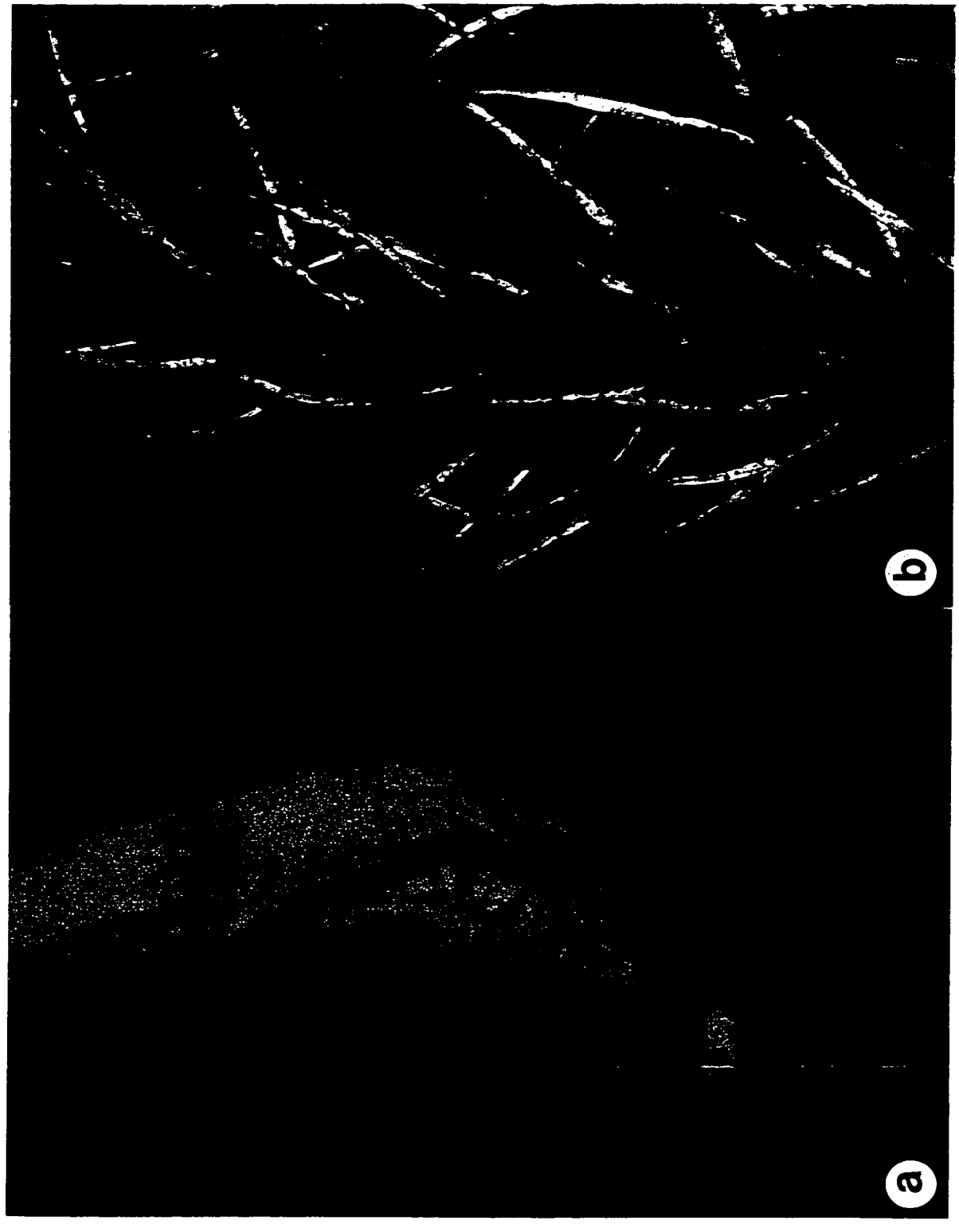


Figure 1-2. Disease cycle of blackleg of canola. Taken from: Canola Growers Manual (Canola Council of Canada, 1989)

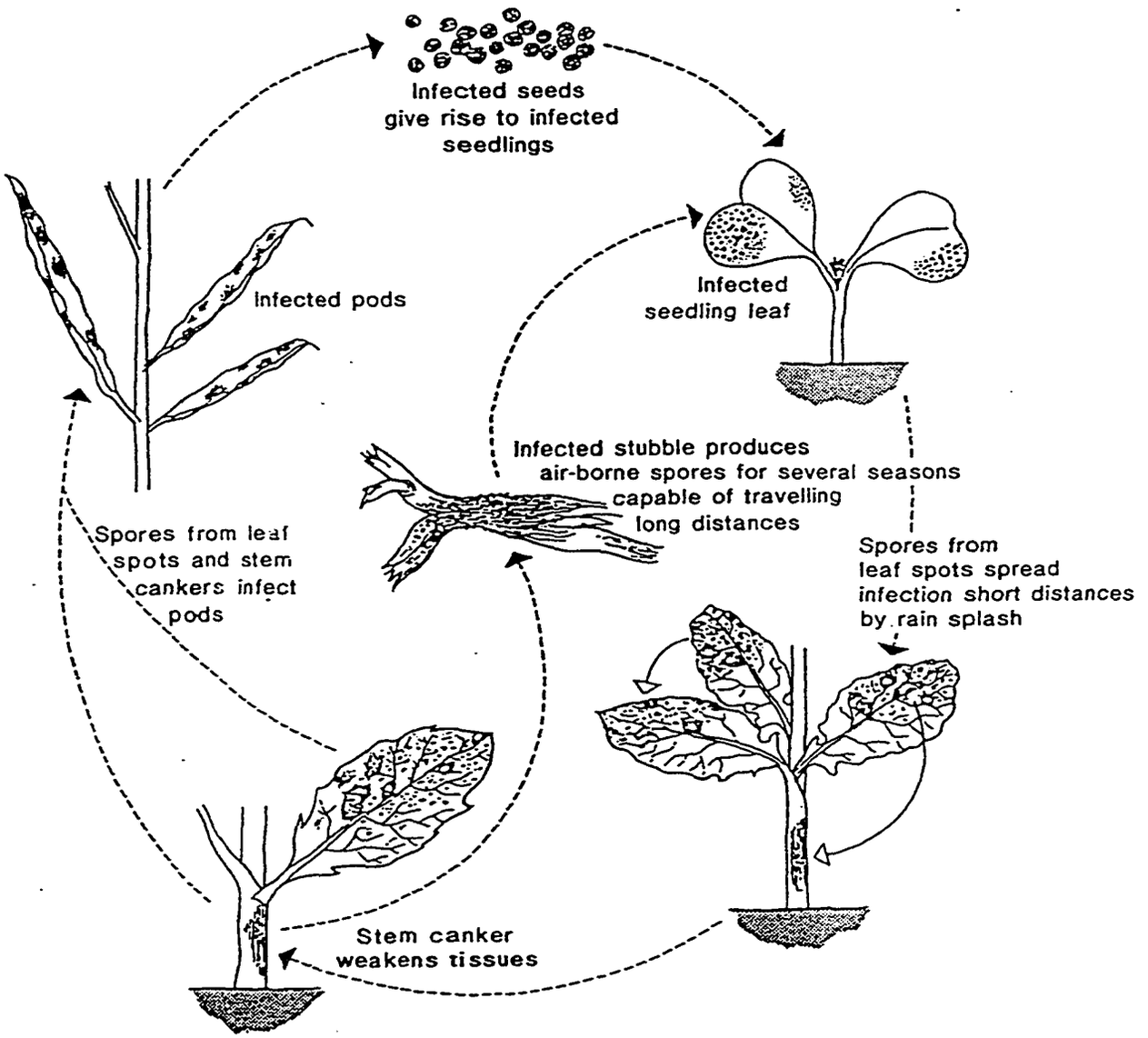


Figure 1-3. *Cyathus olla* infested canola stubble.

(a) *C. olla* infesting canola stubble in the field.

(b) Field collected basidiocarps of *C. olla*.

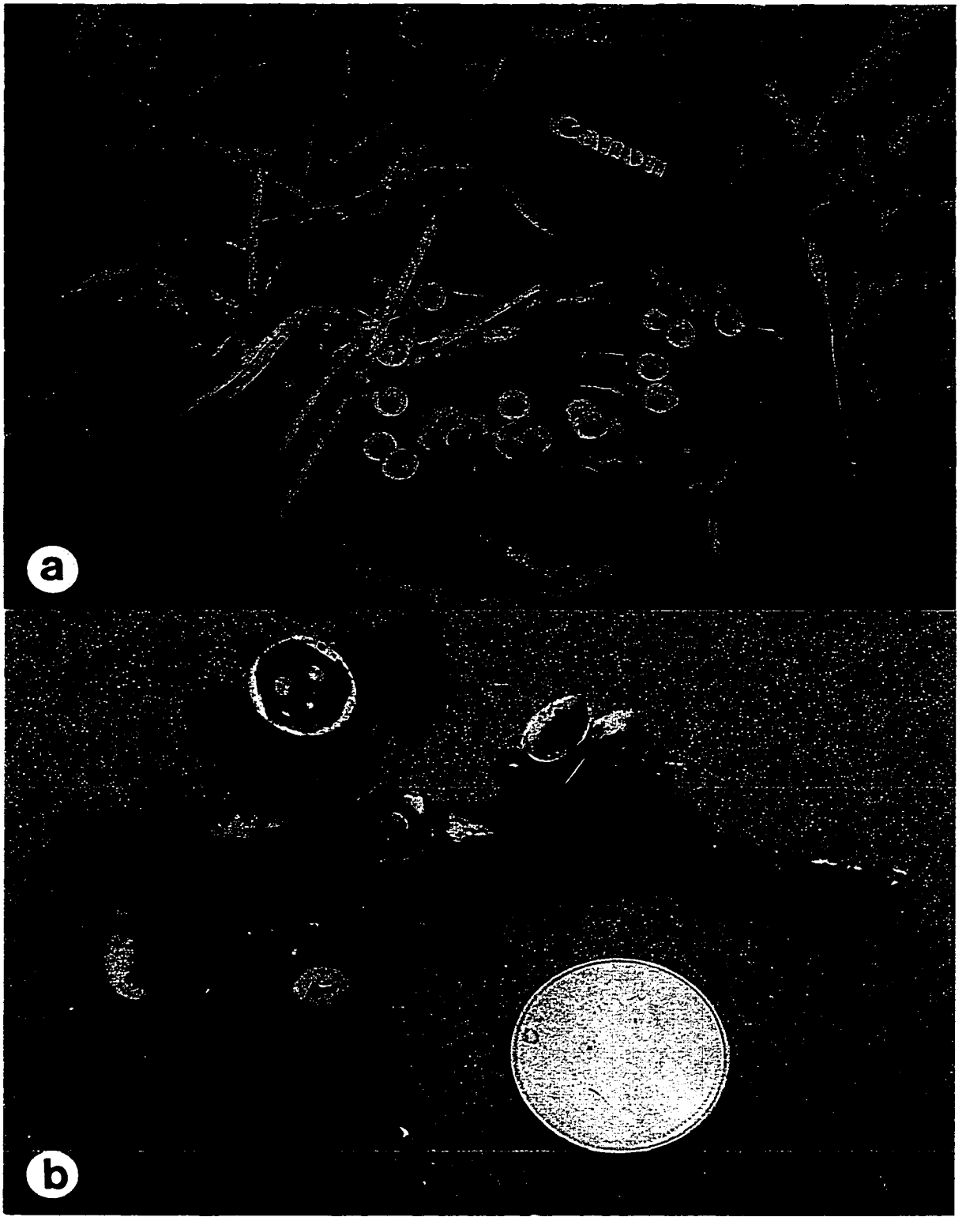


Figure 1-4. Hyphal cords of *Cyathus olla*.

(a) Infested canola stubble with extensive hyphal cord system emanating through the soil.

(b) Hyphal cords colonizing field collected canola stubble.

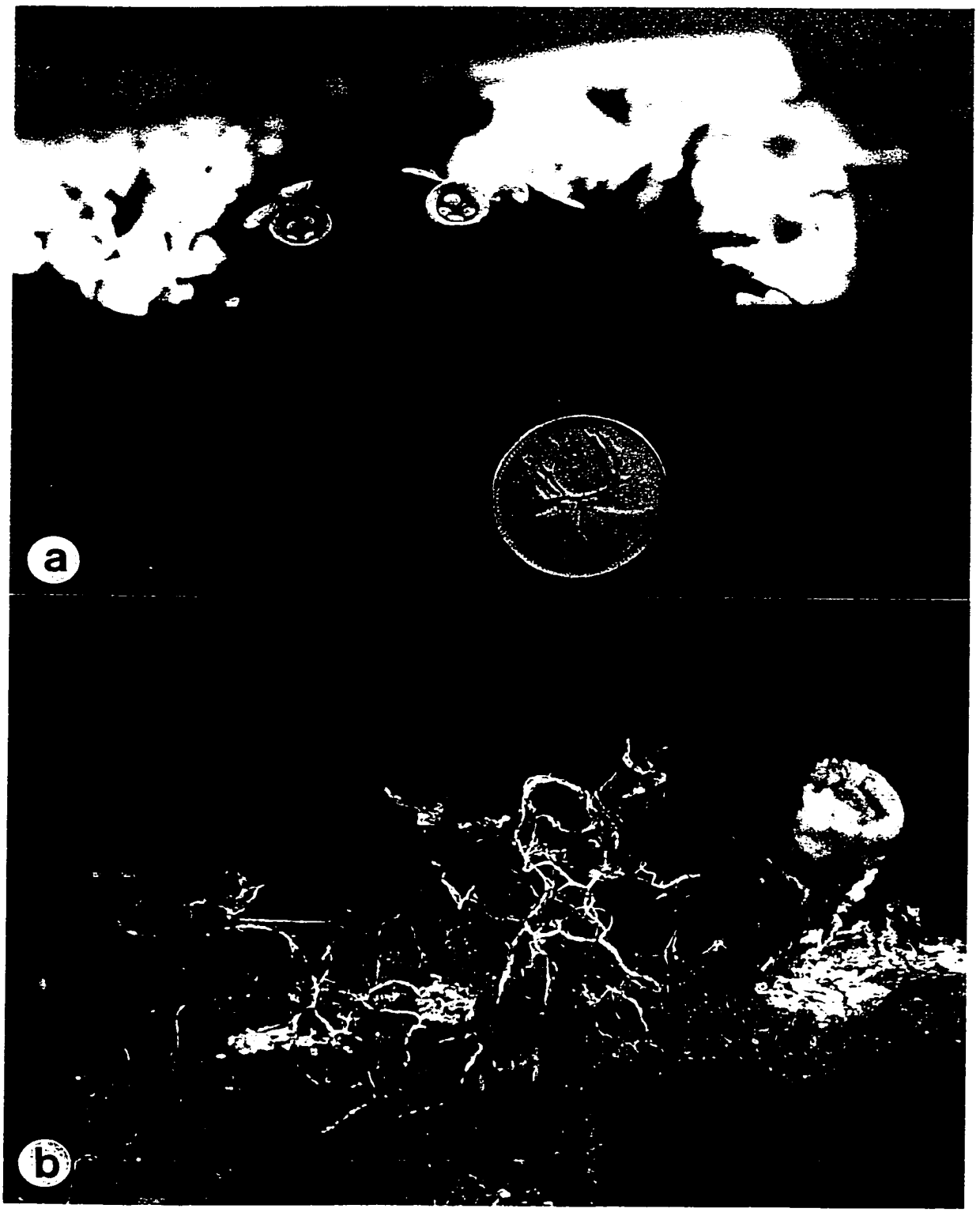
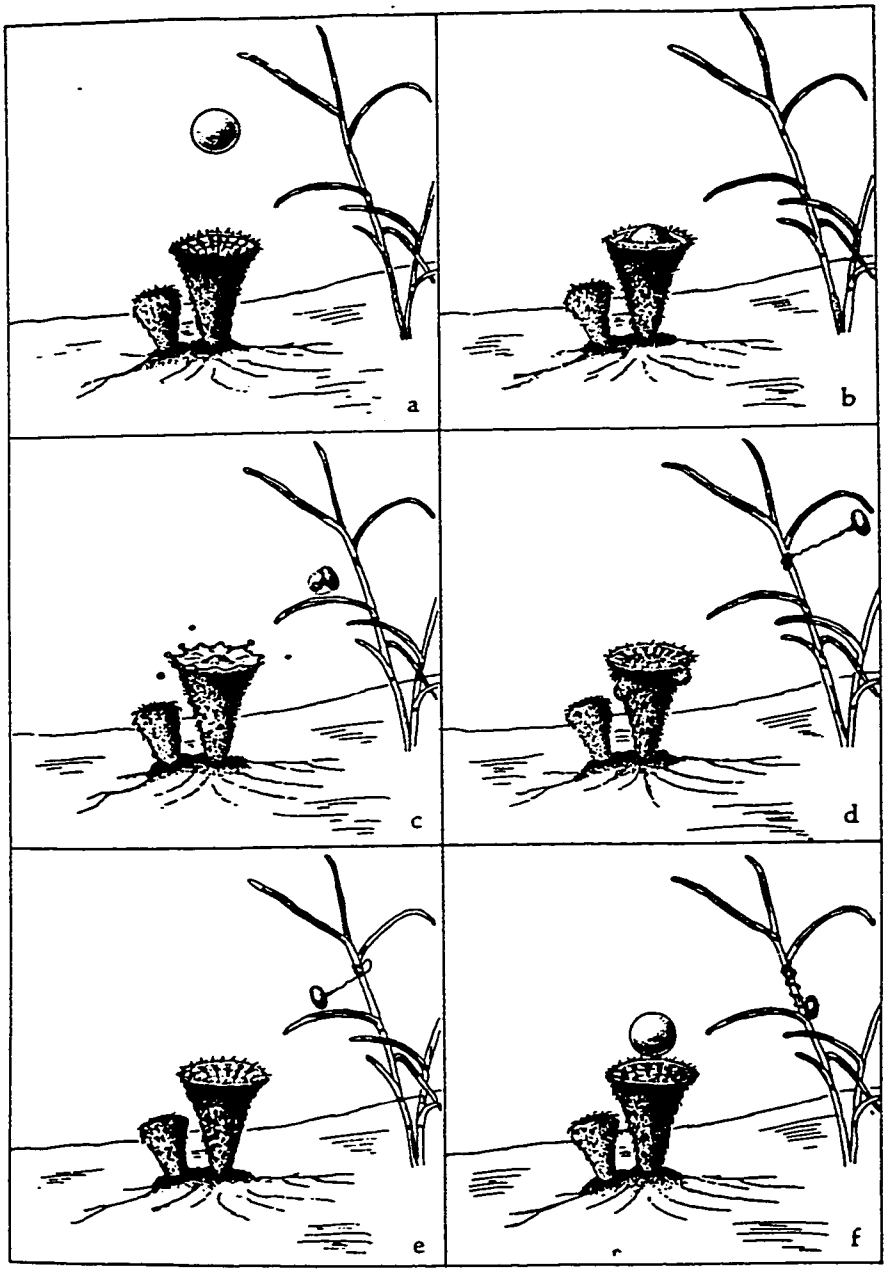


Figure 1-5. Raindrop dispersal mechanism of the Nidulariaceae.

(a, b) Raindrop lands in the basidiocarp, (c) peridiole is ejected and hapteron is exposed, (d) hapteron adheres to a surface, peridiole moves forward and extends funiculus, (e) peridiole is jerked back by limited extension of the funicular cord, (f) peridiole becomes entangled.

Taken from: Can. J. Bot. 29: 224-234. (Brodie, 1951)



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Chapter 2

Production of calcium oxalate crystals by two species of *Cyathus* in culture and infested plant debris [1]

2.1 Introduction

Field infestation of canola stubble by *Cyathus olla* was first reported by Tewari and Briggs (1995). A preliminary study at that time identified calcium rich crystals associated with the colonizing hyphae and hyphal cords on canola stubble, as well as in culture. A thorough chemical investigation was not performed, but the crystals were typical of the dihydrate form of calcium oxalate.

Cyathus striatus (Fig. 2-1) is another species of bird's nest fungus commonly found in Edmonton, Alberta. This species has also been isolated from a woody substratum, and it appears to have a preference for wood chips commonly found as garden or landscaping mulch. Crystal production by *C. striatus* has not been reported, but it is suspected due to its ability to proliferate on a woody substratum.

Many fungi are potent biodegraders of plant debris and contribute significantly to the cycling of nutrients. They produce substantial quantities of organic acids, especially oxalic acid (Connolly and Jellison, 1995; Cromack *et al.*, 1977). Calcium is an essential plant nutrient and is accumulated in appreciable amounts in the higher plants (Demarty *et al.*, 1984; Kirkby and Pilbean, 1984). Plant tissues rich in calcium are often resistant to fungal pathogens. Calcium is incorporated into pectic substances and forms calcium pectate which is resistant to hydrolysis by polygalacturonase (Bateman and Lumsden, 1965). Oxalic acid secreted by fungal pathogens and saprophytes chelates with calcium and removes it from plant cell walls and membrane components (Anagnostakis, 1983), resulting in hydrolysis of pectate by polygalacturonase (Bateman and Beer, 1965) and increased pore size which aids in penetration of cell wall-degrading enzymes. Oxalic acid lowers pH levels to approximately 4.0, optimal for enzymatic acitivity (Bateman and Beer, 1965). Oxalate

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is also thought to play a significant role in the degradation of lignocellulose by regulating certain enzymes involved in the ligninolytic enzyme system (Dutton and Evans, 1996). Accumulations of calcium oxalate crystals on the hyphae of basidiomycetes are commonly reported to be associated with decomposing wood and forest litter (Horner *et al.*, 1995; Connolly and Jellison, 1995; Graustein *et al.*, 1977). Calcium oxalate formation by fungi has recently received much attention due to its importance in pathogenesis in plants, decomposition of plant organic matter, biomineralization, calcium cycling, and general role in the soil environment (Bateman and Beer, 1965; Connolly and Jellison, 1995; Dutton and Evans, 1996; Dutton *et al.*, 1983; Godoy *et al.*, 1990; Horner *et al.*, 1995; Punja and Jenkins, 1984; Rao and Tewari, 1987; Wang and Tewari, 1990).

The present study was undertaken to determine if two common bird's nest fungi, *C. olla* and *C. striatus* produce calcium oxalate crystals during growth on canola stubble and wood chips, respectively, as well as in culture.

2.2 Materials and Methods

2.2.1 Cultures of *C. striatus* and *C. olla*

Cyathus olla was collected on canola stubble (*Brassica* spp.) at the Edmonton Research Station, University of Alberta, Edmonton, Alberta. *Cyathus striatus* was collected on landscaping wood chips at the University of Alberta, Edmonton, Alberta. Cultures from surface sterilized peridiola were grown on V8 juice Rose Bengal agar (V8 juice [Campbell Soup Co.] 200 mL, Rose Bengal 50 mg, Difco Bacto-Agar 20g, $CaCO_3$ 3g, distilled H_2O to 1 liter) and maintained at room temperature (approx. 22°C) in the dark.

2.2.2 Scanning Electron Microscopy and Energy Dispersive X-ray Microanalysis

Agar blocks from 4-wk-old cultures of *C. olla* and *C. striatus*, pieces of canola residue colonized by *C. olla*, and wood chips colonized by *C. striatus* were vapor-fixed with 1% (v/v) osmium tetroxide in water for 4 h, then air-dried overnight at room temperature. Samples were mounted on scanning electron microscope (SEM) stubs and

secured with Marivac colloidal carbon paint. The specimens were sputter-coated with approximately 1 nm layer of gold and examined in a Jeol JSM 6301 FXV SEM operated at 5 or 20 kV. Energy-dispersive X-ray microanalyses were conducted using a Link eXL energy dispersive X-ray system with a light element detector.

2.2.3 Crystal Isolation

To collect crystals, 10 mL of distilled water were added to 20 4-wk-old cultures of *C. olla* and *C. striatus*. The mycelium was scraped from the agar surface and the water and hyphal fragments were collected in a 250 mL Erlenmeyer flask. The slurry was filtered through five sheets of cheesecloth and the filtrate spun at 4000 rpm for 10 min at 20°C in a Sorvall GLC-1 (10 cm rotor) table-top centrifuge. The supernatant was discarded and 10 mL of distilled water were used to resuspend/wash the pellet. The samples were respun and this washing procedure was repeated three times. The pellet was dispersed in 20 mL of distilled water and frozen to await chemical analysis.

2.2.4 FT/ IR and ^{13}C NMR Spectroscopy

The suspension was lyophilized using a Labconco 4.5 Freeze Dryer. This yielded a white crystalline powder that was relatively insoluble in water, hexane, dichloromethane, ethyl acetate, and methanol. An FT/IR spectrum of this powder was obtained using a Nicolet Magna 750 FT/IR with a Nic-Plan IR microscope. The powder was dissolved in deuterium chloride (20% (w/v) solution in D_2O obtained from Aldrich Chemicals) and a ^{13}C NMR spectrum was obtained using a Bruker AM-300 NMR spectrometer (300 Mhz). Calcium oxalate (Fisher Scientific) was used for comparison with the unknown sample.

2.3 Results

In both *C. olla* and *C. striatus*, the hyphae in culture and on natural substrata were present either singly or were organized into mycelial cord-like structures. In both species, many hyphae were heavily encrusted with crystals that rendered them almost unidentifiable. However, in both species most of the superficial aerial hyphae in culture

were virtually free of crystal deposits. Many pieces of canola stubble colonized with *C. olla* were soft and macerated and the xylem elements showed irregularly-shaped holes and other forms of structural damage (Fig. 2-2a). In contrast to this, the wood chips colonized by *C. striatus* did not reveal any macroscopic signs of maceration. Scanning electron micrographs of the same samples showed obvious structural deterioration, with hyphal impressions that were associated with exposed xylem elements, indicative of enzymatic activity.

Cyathus olla in culture and on canola stubble had a compact layer of raphide crystals, approximately 6.0 μm in thickness, present along the length of hyphae (Fig. 2-2b). Styloid crystals were also associated with this fungus but were not as abundant as the raphide type. Styloids and raphides are elongated crystals that are distinguished from each other by their distinctive ends. Both ends of a styloid form a prism, whereas a raphide has a tapered spear-like end. Both these crystal shapes are characteristic of the monohydrate form of calcium oxalate (whewellite) (Frey-Wyssling, 1981). When grown in culture, bipyramidal crystals (approx. 2.5 x 2.5 μm) typical of the dihydrate form of calcium oxalate (weddellite) were immersed in agar and were associated with some hyphae. Most hyphae, however, were heavily encrusted with raphide and styloid crystals.

The crystal morphology was appreciably different in *C. striatus*. When grown in culture, the hyphae of this species were heavily encrusted with bipyramidal (approx. 2.0-6.0 x 2.0-6.0 μm), styloid (approx. 3.0 x 0.5 μm), and variably-shaped crystals (Fig. 2-2c & d). On wood chips, the hyphae were encrusted with raphide druses (spherical aggregates) (approx. 5.5-6.0 μm) which were evenly spaced along the length of individual hyphae (Figs. 2-2e & f).

The FT/IR spectrum of the crystal preparation revealed absorbance bands at 3600-3000 and 1620-1320 cm^{-1} which closely matched those obtained for a sample of calcium oxalate. The ^{13}C NMR spectrum presented a single carbon resonance at 160.9 ppm which was characteristic of the carbonyl carbon resonance for oxalate. On the basis of these spectral data, the crystal preparation was identified as a salt of oxalic acid. X-ray microanalyses revealed a strong peak for the presence of calcium (Figs. 2-3a & b,

Table 2-1). Therefore, the crystals were identified as calcium oxalate on the basis of these three parameters.

2.4 Discussion

This study indicated that *C. olla* and *C. striatus* are crystal-forming fungi and effectively sequester calcium from their substrata, indicating an active role in decomposition. This appears to be the first report of calcium oxalate crystal production by these fungi. Crystal formation can be used as an indicator of biodegradation of natural substrates. Oxalic acid secretion by fungi results in the sequestration of calcium from the cell walls and other components of the substratum (Cromack *et al.*, 1977; Dutton *et al.*, 1993; Rao and Tewari, 1987; Punja and Jenkins, 1984; Bateman and Beer, 1965; Wang and Tewari; 1990). Oxalate acts synergistically with fungal pectinases (Bateman and Beer, 1965). Degradation of calcium pectate results in a weakened wood structure and increased pore size, conducive to further degradation by allowing the penetration of lignocellulolytic enzymes secreted by the fungus (Dutton *et al.*, 1993).

The crystals were identified as being of either the monohydrate or dihydrate form of calcium oxalate. Crystal morphology is influenced by genetics and physiology of the fungus; and potentially by environmental factors such as pH, temperature, and ion availability (Horner *et al.*, 1995; Frey-Wyssling, 1981; Arnott and Webb, 1983). Calcium oxalate dihydrate is the most common form produced by litter-degrading fungi (Connolly *et al.*, 1996; Arnott and Webb, 1983) and other ascomycetes, basidiomycetes, and zygomycetes (Dutton and Evans, 1996). The dihydrate form is more soluble than the monohydrate, but the ecological significance of this is not known.

The crystal encrusted mycelial cord-like structures present on the surface of both the woody substrates and agar plates appear to be similar to rhizomorphs. They have been reported in *C. striatus* (Townsend, 1954), but not in *C. olla*. Rhizomorphs and mycelial cords are commonly formed by basidiomycetes. These linear organs are important for movement and dispersal of fungi through the soil and litter layers (Townsend, 1954), serve as foraging systems for acquisition of nutrient resources (Dowson *et al.*, 1989), and for securing territory by aggressive or combative behavior

(Boddy, 1993). Histological examination and determination of the growing point is required before these structures can be classified as true rhizomorphs or mycelial cords.

Crystal formation is an important quality of *C. olla* and *C. striatus*. Calcium oxalate crystals are a reservoir of calcium for the ecosystem, and more importantly, oxalate in solution increases the effective solubility of iron and aluminum in soil. Oxalate is also a chelator of these two metals, and as such improves the availability of phosphorus for uptake by plant roots (Graustein *et al.*, 1977). Based on these properties, *C. olla* and *C. striatus* may make significant contributions to nutrient cycling and plant nutrition in addition to their active roles as decomposing basidiomycetes.

2.5 Tables and Figures

Table 2-1. Percent composition of elements monitored in crystals from *C. striatus* and *C. olla.*

			%Elemental composition			
	Emission	Energy	*C. olla*		*C. striatus*	
Element	**line**	**range (keV)**	**mean**	**range**	**mean**	**range**
Oxygen	Kα	0.403-0.623	48.46	43.21-54.31	37.92	27.88-51.34
Chlorine	Kα	2.443-2.763	0.76	0.077-1.41	0.53	0.44-0.59
Potassium	Kα	3.123-3.483	0.47	0.33-0.55	0.36	0.27-0.46
Calcium	Kα	3.503-3.863	50.07	44.02-54.90	61.06	47.7-71.05

Means were determined from three replicates.

Figure 2-1. (a, b) Basidiocarps of *Cyathus striatus* on woodchips.

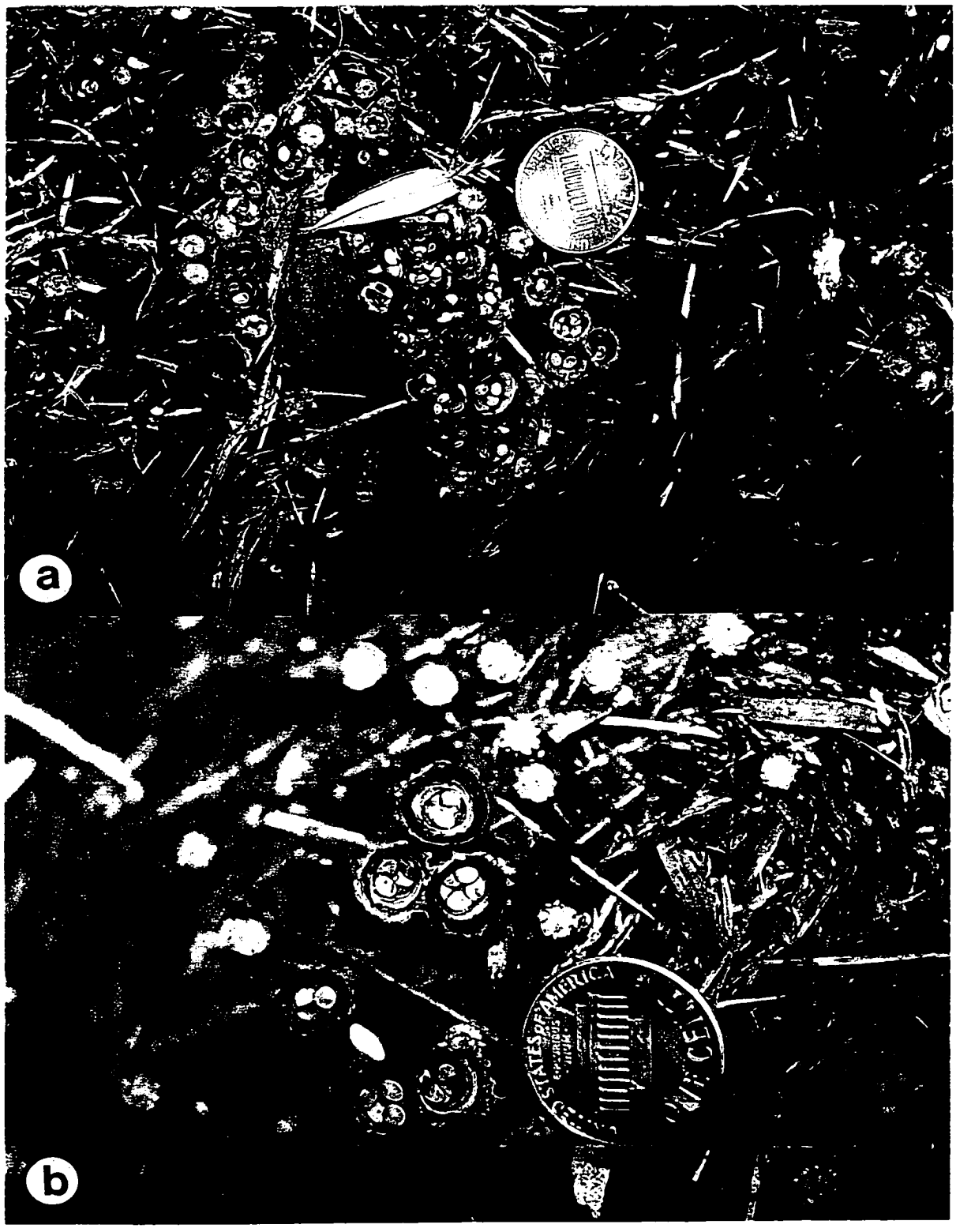


Figure 2-2. Scanning electron micrographs of *Cyathus olla* and *Cyathus striatus*.

(a) Basal stem of canola colonized by *C. olla*. Note the compact layer of raphide crystals on the hyphae. Bar = 10 μm.

(b) *C. olla* hyphae from culture encrusted with raphide, styloid, and bipyramidal crystals. Note the higher magnification of a raphide-encrusted hypha. Bar= 10 μm and 2 μm, respectively.

(c) *C. striatus* hyphae from culture heavily encrusted with bipyramidal crystals. Note that crystal deposition has rendered the hyphae unidentifiable. Bar= 5 μm.

(d) Higher magnification of a bipyramidal and styloid crystals on the hyphae of *C. striatus* from culture. Bar= 1 μm.

(e) Crystal encrusted hyphae of *C. striatus* colonizing wood chips. Note the raphide druses are evenly spaced along the length of hyphae. Bar= 1 μm.

(f) Higher magnification of a raphide druse from (e). Bar= 1 μm.

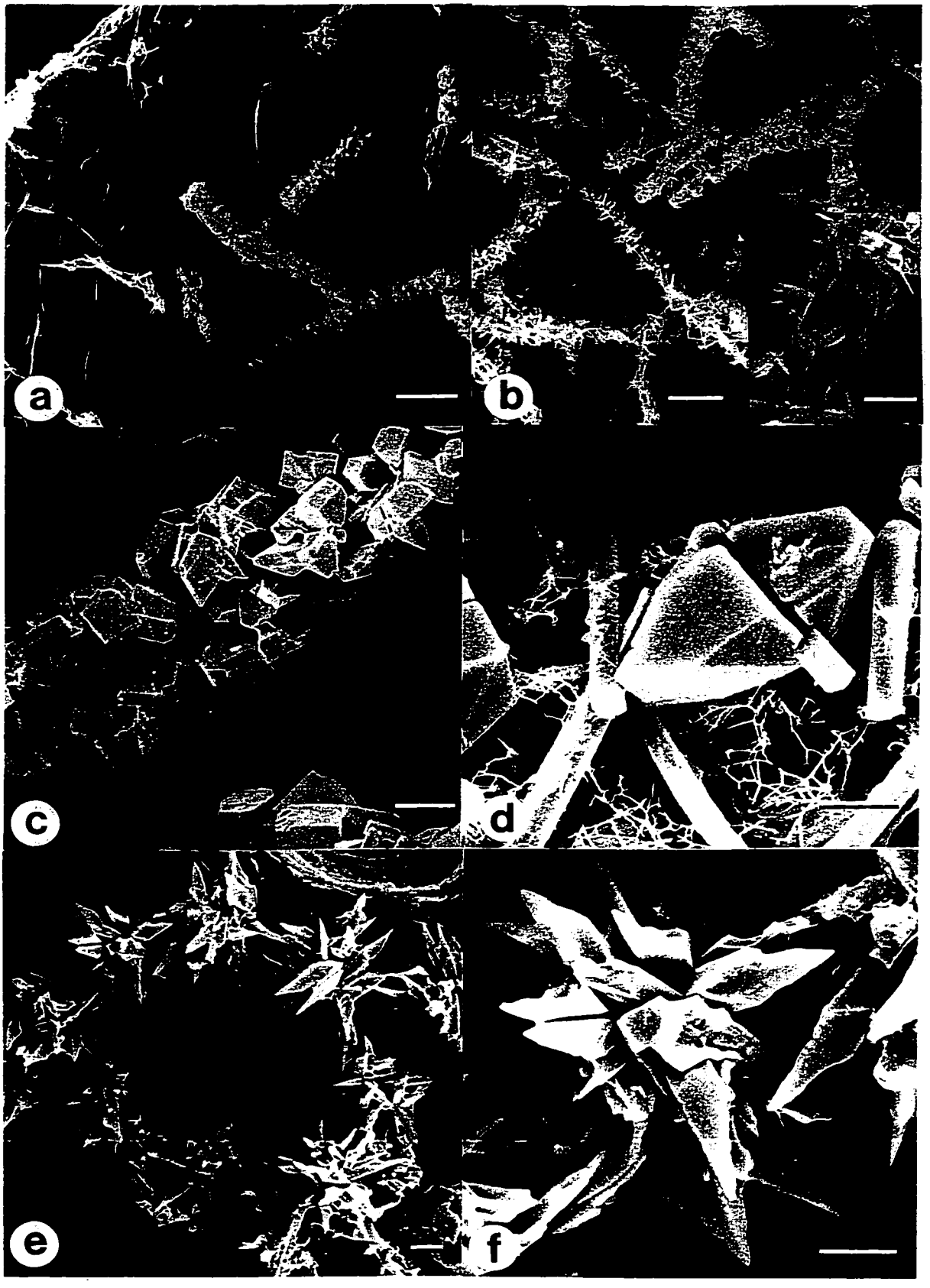
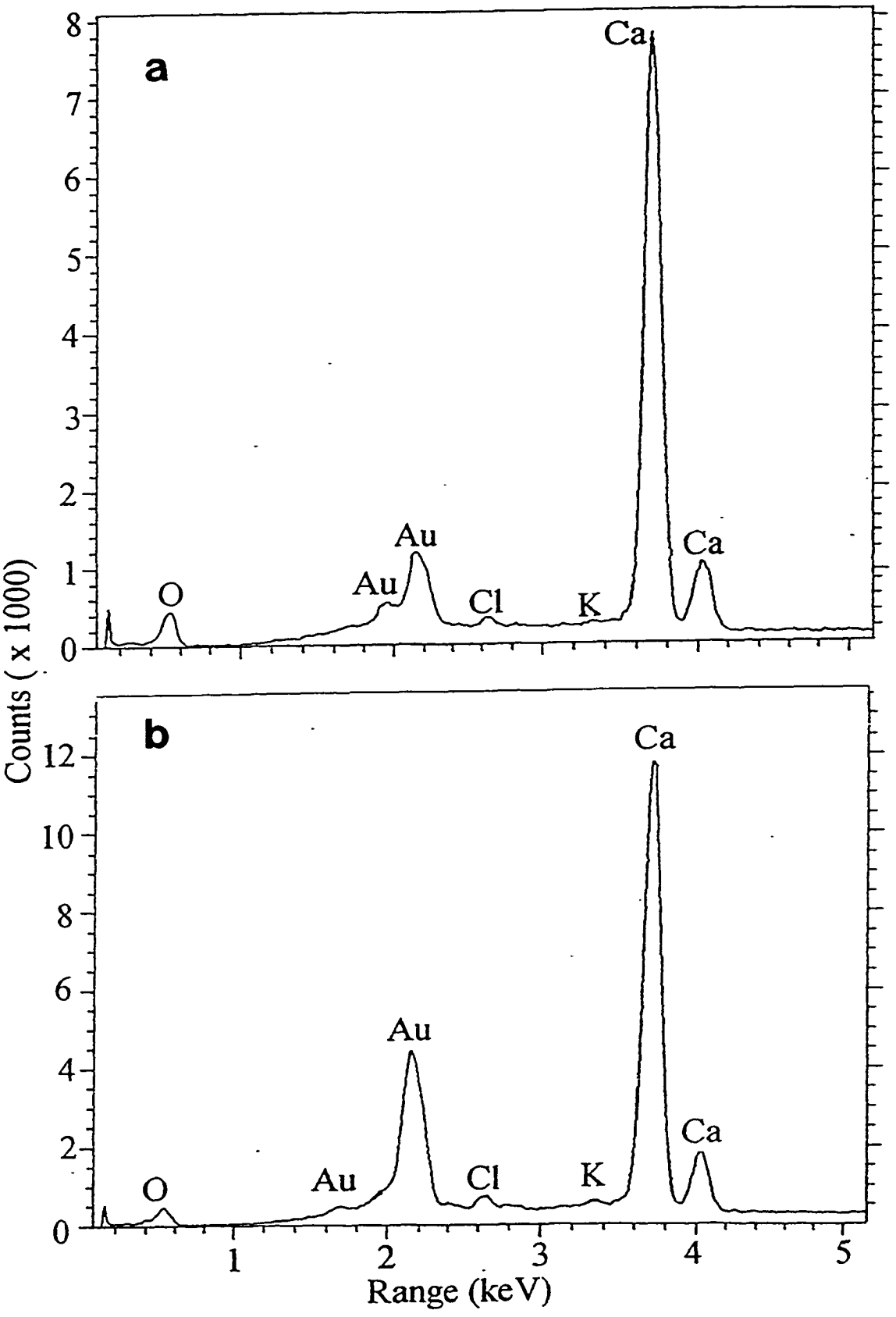


Figure 2-3. X-ray spectra from (a) *Cyathus striatus* and (b) *Cyathus olla.* Note the oxygen and calcium peaks. Gold is present due to the specimen coating, and the chlorine and potassium are detected from the background V8 juice based medium.



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Chapter 3

Biodiversity in crystal production by some bird's nest fungi (Nidulariaceae) in culture[1]

3.1 Introduction

The Nidulariaceae are an interesting group of fungi that have attracted much attention due to their beauty, morphology, and unique dispersal mechanisms. From a functional perspective, these fungi are ecologically important as white-wood rotters involved in the decomposition of numerous substrates worldwide. Some are coprophilous, but many are commonly found growing and fruiting on plant residues, twigs, or some type of plant fiber (Table 3-1) (Brodie, 1975). These fungi are aggressive colonizers due to the formation of mycelial cords that invade the substrate and travel through the soil. In addition to these physical attributes, the Nidulariaceae are a chemically active group that produce many enzymes (Kuhad and Johri, 1991; Orth *et al.*, 1993; Pelaez *et al.*, 1995; Kuhad and Johari, 1987), and an antibiotic complex (Allbutt *et al.*, 1970).

Based on these biochemical and plant residue-colonizing capabilities, research has been initiated to evaluate the potential role of a bird's nest fungus in accelerating biodegradation of crop residues, such as that of canola. Crop stubble serves as a habitat for microbes, and hence plays an important role in the epidemiology of many stubble-borne diseases. Research in this area may lead to the development of a microbial inoculant (biological control agent) to accelerate stubble decomposition, and hence reduce disease incidence.

Recently, calcium oxalate crystals have been found to be associated with the hyphae of *Cyathus olla* and *C. striatus* (Nidulariaceae) (Tewari *et al.*, 1997; Chapter 2). Crystal production is an indicator of oxalic acid biosynthesis and secretion (Dutton *et*

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[1] *A version of this chapter has been published:* Shinners, T.C. and J.P. Tewari. 1997. Canadian Journal of Chemistry 75: 850-856.

al., 1993; Punja and Jenkins, 1984; Rao and Tewari, 1987), and suggests an active role in enzymatic biodegradation. Crystal-forming fungi are important ecosystem inhabitants as they are involved in calcium-recycling, nutrient availability for plant growth, and soil genesis (Graustein *et al.*, 1977; Horner *et al.*, 1995). Production of crystals by *C. olla* and *C. striatus* supports their role as decomposers, but other members of the Nidulariaceae have not been studied with respect to calcium oxalate crystal production.

Crystals have been described on the hyphae of many members of Basidiomycotina under a given set of conditions (Dutton *et al.*, 1993; Horner *et al.*, 1995; Whitney and Arnott; 1987; Arnott and Webb, 1983). However, it is not known if crystal morphology would vary if the conditions were changed. Cultures of bird's nest fungi are usually mycelial and fruiting can only be achieved in some cases under special conditions, often requiring extended periods of time. It would be advantageous to develop diagnostic criteria for species identification using hyphal and crystal morphology.

As a continuation and expansion of the study by Tewari *et al.* (1997), hyphae of many species of Nidulariaceae were examined to survey crystal production within this group of fungi. The objectives of this experiment were to determine if various species of bird's nest fungi produce different types of crystals, if crystal morphology was affected by growth media, and if species from various geographical areas and habitats differed in crystal production.

3.2 Materials and Methods

3.2.1 Fungal Cultures

Fungal cultures were obtained from the University of Alberta Microfungus Collection, Edmonton, and the National Mycological Herbarium, Agriculture & Agri-Food Canada, Ottawa (Table 3-2). All cultures were subcultured on to V8 agar (V8 juice [Campbell Soup Co.] 200 mL, Difco Bacto-Agar 20 g, $CaCO_3$ 3 g, distilled H_2O to 1 liter) and Difco potato-dextrose agar (PDA) and maintained at room temperature (approx. 22°C) in the dark.

3.2.2 Scanning Electron Microscopy and Energy Dispersive X-ray Microanalysis

Agar blocks of both V8 and PDA media were excised approximately 1 cm from the original inoculation plug from 3-wk-old cultures of *Nidula niveo-tomentosa, Crucibulum laeve, Cyathus olla, C. earlei, C. pygmaeus, C. canna, C. africanus, C. pallidus, C. julietae, C. stercoreus, C. striatus, C. berkeleyanus,* and *C. bulleri.* Each block was placed on a microscope coverslip (7 mm diameter) and secured with double-sided tape. All samples were vapor-fixed with 1% (v/v) osmium tetroxide in water for 1 h, then air-dried for several days in a fume hood. Samples were mounted onto scanning electron microscope (SEM) stubs and secured with Marivac colloidal carbon paint. The specimens were sputter-coated with approx. 1 nm layer of gold and examined in a Jeol JSM 6301 FXV SEM operated at 5 kV. Energy dispersive X-ray microanalyses were conducted using a Link eXL energy dispersive X-ray system with a light element detector. All samples were examined in triplicate.

3.2.3 X-ray Diffraction Analysis

Crystals for X-ray diffraction analysis were collected from a 3-wk-old culture of *C. striatus.* A hyphal suspension was prepared by adding 10 mL of distilled water to the agar plate, and the mycelium was scraped from the surface. The suspension was filtered through four layers of cheesecloth and the filtrate spun at 1750g for 10 min at 20 °C in a Sorvall GLC-1 table-top centrifuge. The supernatant was discarded and the pellet was resuspended in 5 mL of distilled water. Droplets of the solution were dried down on a quartz plate and examined using a Rigaku-Geigerflex vertical goniometer system with a graphite monochromator. The crystals were exposed to cobalt radiation ($CoK_{\alpha\ 1+2}$ = 1.79026 Å) and compared to American Society for Materials (ASTM) X-ray standard file cards for weddellite (17-541) and whewellite (20-231).

3.3 Results

In total, 13 species of bird's nest fungi were examined. From this group, 10 were shown to produce crystals rich in calcium. Detailed crystal morphology for each species is presented in Table 3-3. Crystals were not observed in *C. pygmaeus, N. niveo-*

tomentosa, and *C. laeve*. Hyphae of these species appeared "clean" (Fig. 3-1a). In contrast to this, hyphae of *C. earlei, C. stercoreus,* and *C. africanus* (Fig. 3-1b) growing on V8 media were heavily encrusted with variably-shaped crystals, typical of both the monohydrate (whewellite) and dihydrate (weddellite) forms of calcium oxalate (Frey-Wyssling, 1981). Encrustation in these species was extremely heavy, rendering hyphae unidentifiable. On PDA, crystals were evident on these species but were considerably sparser compared with hyphae on V8 agar.

Cyathus olla, C. striatus, C. berkeleyanus, C. julietae, C. bulleri, and *C. pallidus* all produced crystals of various morphologies when grown on V8 media. From this group, only *C. olla* and *C. striatus* produced crystals on PDA. *Cyathus canna* was an exception, producing styloids and variably shaped crystals on PDA and none when grown on V8 agar. Crystals were observed in druses (spherical aggregates) (Fig. 3-1c), or randomly attached to hyphae singly or in groups. Druses produced by *C. olla* and *C. berkeleyanus* on V8 agar were all raphide crystals, but other crystal clusters seen on these species, as well as on many others, contained many different crystal types (Fig. 3-1d).

Crystal types observed during this study were typically styloid- (Fig. 3-1e) and raphide-shaped (monohydrate forms of calcium oxalate) (Fig. 3-1f), or bipyramidal crystals, typical of the dihydrate form (Fig. 3-1g). Styloids and raphides are elongated crystals that are easily distinguished from one another. Both ends of a styloid crystal form a prism, whereas raphide crystals form a tapered, spear-like end (Frey-Wyssling, 1981). Dihydrate forms such as bipyramidal types have a four-fold axis, a plane of symmetry perpendicular to it, and a center of symmetry with no symmetrical planes parallel to the crystal axis (Frey-Wyssling, 1981).

Energy dispersive X-ray microanalyses of all crystal types produced spectra high in calcium (Fig. 3-2). Osmium and gold peaks resulted from the fixative and gold coating, respectively. Potassium and chlorine peaks are attributed to components from the V8 juice based medium. X-ray diffraction results of the crystals from *C. striatus* confirmed the presence of both the monohydrate (whewellite) and dihydrate (weddellite) forms of calcium oxalate (Fig. 3-3). Based on these spectral data, documented calcium oxalate

crystal morphology, and previous identification of calcium oxalate crystals associated with *C. olla* and *C. striatus* (Tewari *et al.,* 1997), the crystals observed in this study were identified as calcium oxalate.

3.4 Discussion

Crystal production and morphology may be influenced by many factors including oxalic acid secretion, calcium availability, hydration state, and other physical and chemical environmental factors (Punja and Jenkins, 1984; Frey-Wyssling, 1981; Arnott and Webb, 1983; Horner *et al.,* 1995). Diversity among species of Nidulariaceae with respect to crystal production appears to be attributed to species-specific variation since factors such as calcium availability and environmental conditions were consistent throughout this experiment. In many plants, calcium oxalate crystal shape and location are species and tissue specific (Horner and Wagner, 1995). There are many similar reports for fungi (Arnott, 1995); however, with respect to the Nidulariaceae, many more isolates must be examined to confirm these observations.

Crystal production was greatest on the V8 medium (approximately 3.01 g Ca/ L) which had a greater concentration and availability of calcium than PDA (approximately 30.85 - 38.85 mg Ca/ L). Calculations of calcium content are based on media formulation information and mineral analyses provided by Campbell's Soup Company, Difco Laboratories, and potato mineral content information (Smith, 1968). The approximate calcium content of PDA does not reflect the actual value since PDA is made from potato infusion. For this preparation, the solid phase, which contains most of the calcium (plant cell walls), is removed. Therefore, the actual value would be significantly lower than the estimate. From this information, it can be assumed that calcium was a limiting factor to crystal production on the PDA medium for most species. It is not clear why *C. canna* produced crystals on PDA and none on the V8 medium. This species may require a starch-rich medium for induction of crystals, but this stipulation has to be validated further.

Morphological variation of crystals is a function of hydration state and was species and medium specific. There was no association between crystal types and

geographic and habitat distribution of species. *Cyathus stercoreus* is coprophilous whereas all the other species used in this investigation were from woody substrata. These two groups did not differ in crystal morphologies.

Crystal-producing ability of the fungi observed in culture can be extrapolated to natural environments and the ecological niche these fungi inhabit. Many bird's nest fungi are involved in the decomposition of plant residues. Oxalic acid secretion and subsequent sequestration of calcium weakens cell wall stability, and improves access for enzymes involved in decomposition (Dutton *et al.*, 1993; Punja and Jenkins, 1984). Oxalate may also play a role in regulating the ligninolytic enzyme system (Dutton and Evans, 1996). Horner *et al.* (1995) have suggested that crystal-forming fungi are ecologically important for calcium-recycling, nutrient availability, and soil genesis. From this study, it can be concluded that many species of the Nidulariaceae play a role in these environmental/ ecological processes. Crystals were not observed in all species examined in this experiment, however, the importance of these species cannot be minimized by these results. Crystal production by these fungi may be induced by natural or environmental factors not provided in this experiment.

3.5 Tables and Figures

Table 3-1. Geographic location and common substrates of some members of the Nidulariaceae.

Species	Location	Common Substrate
Crucibulum laeve	-Europe -North America -South America -Japan -Australia/ New Zealand -(temperate zone species)	-Stems, twigs, old nut shells, wood fruits, woodchips, manure -Primarily lignicolous
Cyathus africanus	-Tanzania	-Unknown
C. berkeleyanus	-Widespread in tropics	-Bamboo pots
C. bulleri	-Tropics	-Unknown
C. canna	-Exclusively tropical	-Unknown
C. earlei	-Tropical/ sub-tropical	-Unknown
C. julietae	-Jamaica	-Unknown
C. olla	-Europe -North America -South America -South Africa -Australia -Not found in warm, moist tropics	-Old wood, dead herbaceous plant stems -Plant fibers/ artifacts
C. pallidus	-American tropics -West Indies -Mexico -South America -China	-Small chips and twigs -Compost/ mulch
C. pygmaeus	-United States	-Old dead stems of shrubby plants of arid area -Manure
C. stercoreus	-World-wide	-Coprophilous -Manured soils

C. striatus	-Widespread in temperate areas	-Small twigs, woody leaf mould -Cereal residue -Plant fiber/ artifacts
Nidula niveo-tomentosa	-Western North America -South America -Japan -New Zealand -Jamaica	-Associated with bracken fern

Table 3-2. Sample source and identification

Isolate	ID #
Crucibulum laeve	DAOM 196687[a]
Cyathus africanus	DAOM 196686[a]
Cyathus berkeleyanus	DAOM 197568[a]
Cyathus bulleri	DAOM 195062[a]
Cyathus canna	DAOM 196684[a]
Cyathus earlei	UAMH 8104[b]
Cyathus julietae	DAOM 196681[a]
Cyathus olla	UAMH 8276[b]
Cyathus pallidus	DAOM 196678[a]
Cyathus pygmaeus	UAMH 8020[b]
Cyathus stercoreus	DAOM 172472[a]
Cyathus striatus	USA[c]
Nidula niveo-tomentosa	UAMH 8101[b]

[a]Received from the National Mycological Herbarium of Canada

[b]Received from the University of Alberta Microfungus Collection

[c]Collected by I. Tewari

Table 3-3. Crystal morphology on PDA and V8 media.

Species	PDA	V8	Comments
Crucibulum laeve	None	None	
C. africanus	Raphide,	Raphide, styloid, bipyramidal, variable	Hyphae on V8 hyphae heavily encrusted
C. berkeleyanus	None	Styloid, raphides, bipyramidal, variable	
C. bulleri	None	Styloid, raphide	
C. canna	Raphide, variable	None	
C. earlei	Raphides Styloid	Raphides, bipyramidal, variable	Hyphae completely encrusted
C. julietae	None	Raphide, styloid	Hyphae completely encrusted
C. olla	Raphides	Bipyramidal	
C. pallidus	None	Styloid	Dense mycelial mat
C. pygmaeus	None	None	
C. stercoreus	Styloid	Styloid, bipyramidal, variable	Hyphae on PDA were completely encrusted
C. striatus	Styloid, raphide	Raphide, styloid, bipyramidal, variable	
N. niveo-tomentosa	None	None	

Figure 3-1. Scanning electron micrographs of calcium oxalate crystals associated with *Cyathus* spp.

(a) Crystal free hyphae of *C. pygmaeus*. Bar= 5 μm.

(b) Heavily encrusted hyphae of *C. africanus*. Bar= 5 μm.

(c) Raphide druses produced by *C. olla* on PDA. Bar= 1 μm.

(d) *C. stercoreus* hyphae encrusted with various crystal types. Bar= 2 μm.

(e) Typical styloid crystal shapes. Bar= 1 μm.

(f) Typical raphide crystals. Note the spear-like, tapered ends. Bar= 1 μm.

(g) Crystals typical of the dihydrate form of calcium oxalate. Note the bipyramidal ends. Bar= 1 μm.

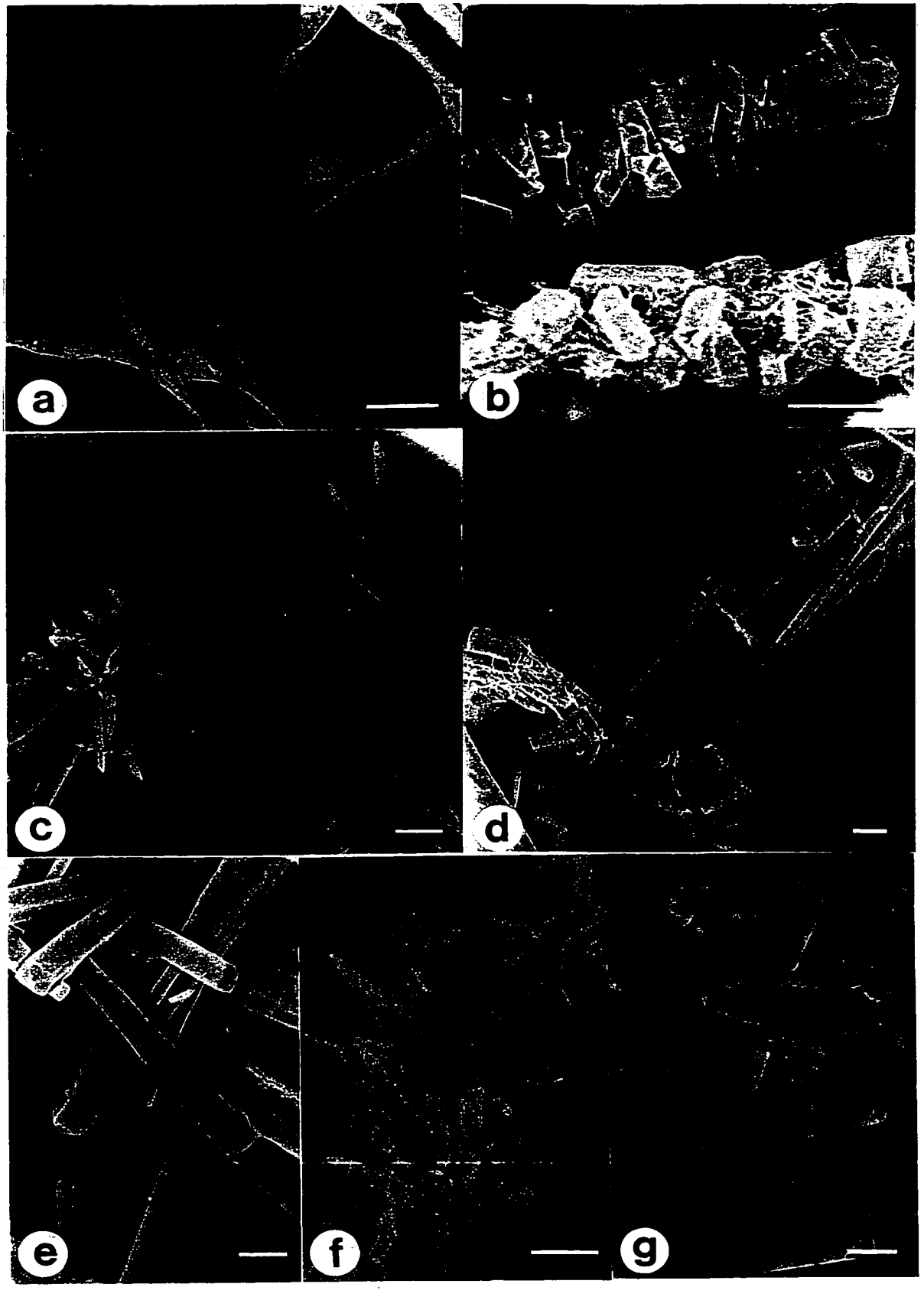


Figure 3-2. X-ray spectrum of crystals from *Cyathus earlei*. Note the calcium peaks.

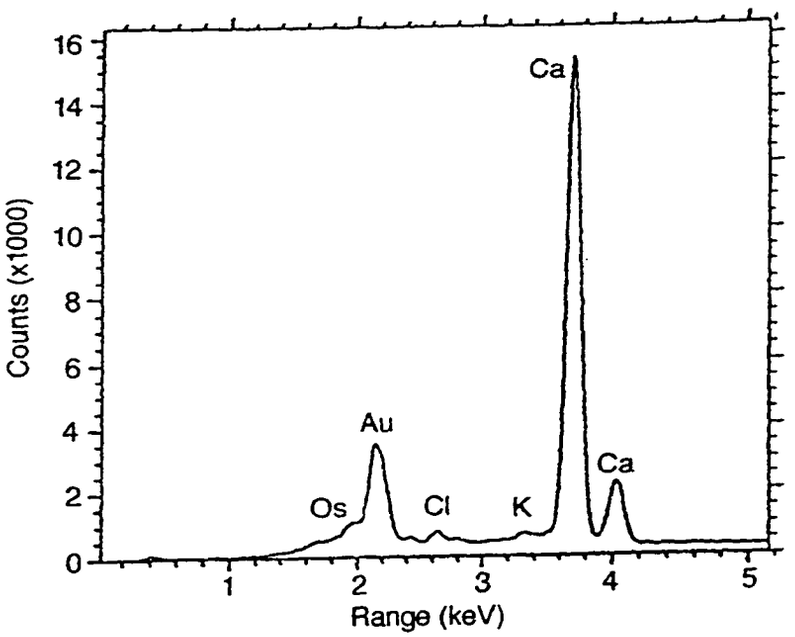
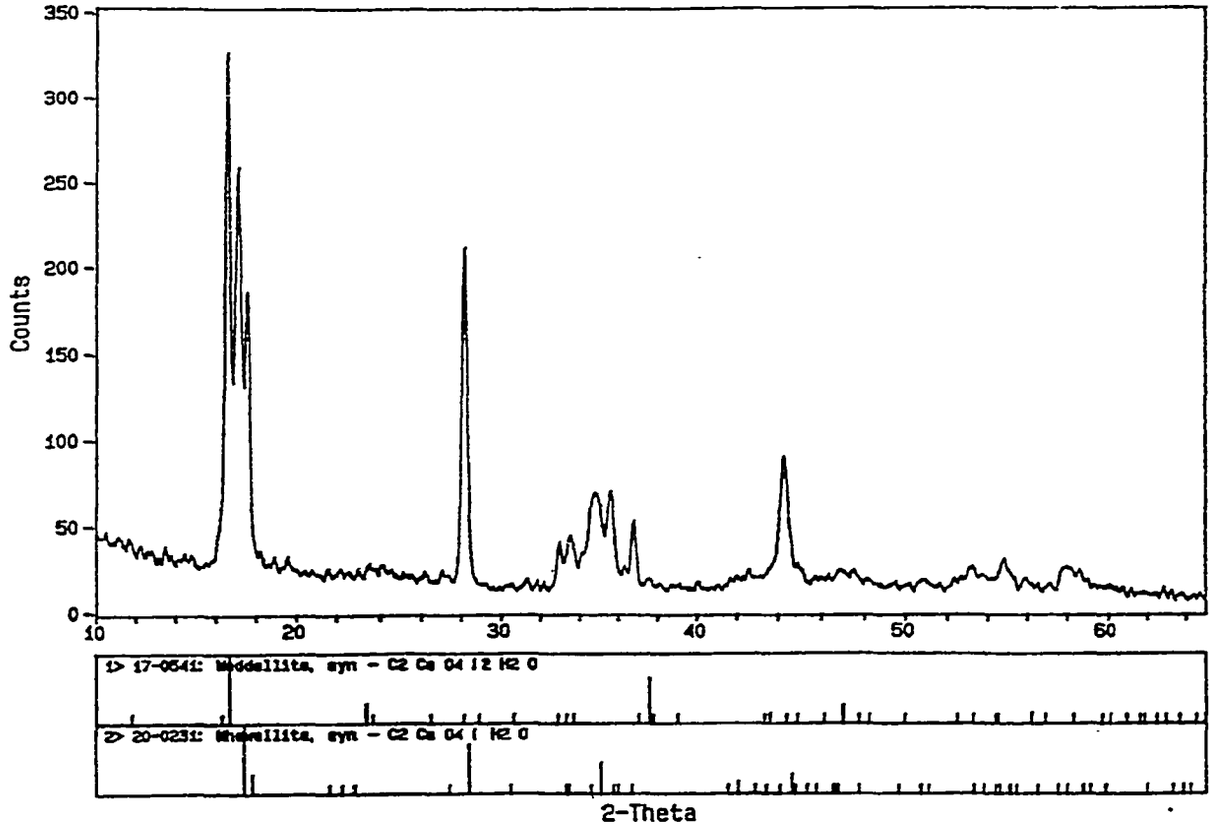


Figure 3-3. X-ray diffraction spectrum of crystals from *Cyathus striatus*. Note the spectral comparison to ASTM standard cards for weddellite and whewellite.



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Chapter 4

Morphological and RAPD analyses of *Cyathus olla* from crop residue[1]

4.1 Introduction

The biology of the bird's nest fungi has been studied in detail by Brodie (1975), but the literature contains very few accounts of this group in applied research. For this reason, accessions were collected or acquired and research was initiated to examine the biology and canola stubble colonizing and decomposing capabilities of *Cyathus olla*. Variability in this species was noted by Brodie, but only three forms, *C. olla* f. *olla*, *C. olla* f. *anglicus*, and *C. olla* f. *lanatus* were recognized (Brodie, 1952, 1978). Morphological and genetic variation that may exist within the species *C. olla* needs to be studied further as this information is critical for any future research and development as a biological control agent, since variability may also exist with respect to biochemical and ecological capabilities involved in stubble decomposition.

The development of random amplified polymorphic DNA (RAPD) analysis by Welsh and McClelland (1990) has provided a method for assessing variation and genetic relatedness within species. A comparison between polymorphisms in genomic fingerprints produced by this method is sufficient to distinguish between strains (Welsh and McClelland, 1990). This method is relatively fast and no prior knowledge of genetic information is required. Since its development, PCR-based RAPD analysis has been used extensively for analyzing plant and fungal genetics (Weising *et al.*, 1995; Fabbri *et al.*, 1995; Yu and Nguyen, 1994; Patwary *et al.*, 1993; Pooler and Hartung, 1995; Van Coppenolle *et al.*, 1993; Yoon and Glawe, 1993). To date, no molecular work on the bird's nest fungi has been documented in the literature. The objectives of this study were to assess the variability of *C. olla* accessions on the basis of basidiocarp

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[1] *A version of this chapter has been published:* Shinners, T.C. and J.P. Tewari. 1998. Mycologia 90: 980-989.

morphology, cultural characteristics, and PCR-based RAPD analysis and to use this molecular tool as an indicator of genetic similarity.

4.2 Materials and Methods

4.2.1 Accessions

In total, 43 *C. olla* accessions were used in this study, many of which were collected as basidiocarps, or obtained as mycelial cultures from culture collections (Table 4-1). All basidiocarps, with the exception of accession #4 and #6 were collected on canola or cereal residue from agricultural fields. The basidiocarps were examined and classified according to the taxonomic key of Brodie (1977). Data were collected on 12 morphological characters including basidiocarp height, width, outer color and texture (smooth or hairy), inner color and texture (smooth, concentric circles, or plicate) and shape of the mouth (broad, sulcate or flared); peridiole size, shape and color; and basidiospore size and shape. Morphological dissimilarity values were calculated for accession #1-26, #32-43 and transferred to a data matrix (38 x 38) and subjected to unweighted pair group method of arithmetic averages (UPGMA) analysis using the NTSYS-pc software package (Exeter software, Setauket, New York). Basidiocarps collected during this study were compared with *C. olla* and plicate *Cyathus* spp. (Table 4-2) borrowed from the National Mycological Herbarium of Canada.

Mycelial cultures were obtained by surface sterilizing peridioles in 1% (v/v) sodium hypochlorite for 1 min followed by a sterile distilled H_2O wash. The peridioles were cut, plated on to V8 juice rose bengal agar [V8 juice (Campbell Soup Co.) 200 mL/ L, Bacto agar (Difco) 20 g/ L, $CaCO_3$ 3.0 g/ L, 0.004% rose bengal], swabbed with streptomycin sulfate, and then incubated at 21°C in the dark.

All accessions were grown on both V8 rose bengal and potato dextrose agars (PDA) and maintained under the previously mentioned conditions. Colony growth characteristics were recorded regularly. Mycelium for DNA extraction was cultured in 50 mL volumes of liquid V8 juice rose bengal medium and maintained at room temperature (21°C).

4.2.2 DNA extraction

Approximately 0.3 g of mycelium from 8-10 d old cultures of *C. olla* was harvested and placed into 1.5 mL microfuge tubes. The mycelium was washed in 1XTE buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, pH 8.0), and then ground in the extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) using liquid nitrogen and a mechanical conical grinder. This crude preparation was digested with RNase A (Sigma) (10 mg/ mL) at 37°C for 1 h, followed by a phenol: chloroform: isoamyl alcohol (25:24:1) extraction, and then a chloroform: isoamyl alcohol (24:1) extraction. Sodium acetate (3 M) was added prior to DNA precipitation with isopropanol. The resulting pellet was washed in 70% ethanol, dried, and dissolved in 50 µL of 1XTE buffer. The integrity of DNA was ascertained by electrophoresis on a 1% (w/v) agarose gel. All samples were stored at –20°C until further use. This method is similar to that of Weising *et al.* (1995), however, modifications were made for the removal of RNA and some proteins. In addition to *C. olla,* DNA from nine *C. striatus* accessions (Table 4-3) was also extracted and amplified for RAPD fingerprinting comparisons since a few *C. olla* isolates displayed some morphological features typical of *C. striatus.*

4.2.3 PCR amplification

Twenty primers (Kit A) of 10 arbitrary nucleotides each (Operon Technologies Inc., Alameda, California) were used in a preliminary screening experiment. Based on resolution and reproducibility of the amplification patterns produced, 13 primers were chosen for use in this study (Table 4-4). PCR amplification reactions were performed in 25 µL volumes that contained 1-1.5 unit Taq polymerase (Gibco-BRL, Life Technologies, Inc., Gaithersberg, Maryland), dNTP mix (0.2 mM each of dCTP, dGTP, dATP, and dTTP), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ (Gibco-BRL), 1 µL primer, and 100-150 ng of genomic DNA. The mixture was vortexed and centrifuged briefly, then overlaid with 50 µL of paraffin oil.

The thermocycler (Thermolyne, Temp. Tronic, Barnstead/ Thermolyne Corporation, Dubuque, Iowa) was programmed for denaturation of DNA for 5 min at 95°C, followed by 40 cycles of 95°C for 1 min, low stringency primer annealing at 35°C

for 1 min, and an extension step at 72°C for 1 min. The program was completed with an additional step of 72°C for 5 min (Sharma and Tewari, 1998). Following amplification, the products were electrophoresed on a 1% (w/v) agarose gel in 0.5X Tris Borate EDTA (TBE) buffer at 120 V for approximately 2 h. A 1 Kb DNA ladder (Gibco) was run for molecular weight size comparison. Gels were stained with ethidium bromide for 15 min, and destained in sterile water also for the same time. Following this procedure, gels were visualized with UV light and photographed with Polaroid 57 or 55 film or recorded using Gel Doc Molecular Analyst (BioRad) software for Macintosh.

4.2.4 Statistical analysis

Results generated from the RAPD analyses were scored based on RAPD markers exhibited by the accessions being compared. Dissimilarity coefficients (genetic distances) of the accessions were calculated based on D= 1-S, where D is defined as dissimilarity, and S = similarity. The Nei similarity index, $S= 2N_{ab} / (n_a + n_b)$ was used, where N_{ab}= number of shared amplification fragments with the same molecular weight between accessions a and b; n_a = the number of scored amplicons for accession a, and n_b = the number of scored amplicons of accession b (Nei and Li, 1979). Dissimilarity values range from 0 to 1, with 1 being dissimilar and 0 indicating similarity. The calculation of dissimilarity, or genetic distance, was used to compensate for the limitation often imposed on RAPD data by using similarity values. Comigrating bands that do not represent the same sequence, variation in amplification products, or length mutations in amplified regions can result in overestimation or underestimation of similarity (Yoon and Glawe, 1993). For these reasons, dissimilarity was the preferred coefficient.

Dissimilarity values were used to construct a 43 x 43 matrix. The data were subjected to UPGMA analysis using the NTSYS-pc software package to produce a dendrogram. All possible ties were accounted for (Backeljau *et al.*, 1996), and the goodness of fit, or cophenetic correlation coefficient was determined using the MXCOMP program.

4.3 Results

4.3.1 Basidiocarp morphology

All accessions of *C. olla* obtained as basidiocarps were examined and major characteristics were recorded. Variation within a species is naturally expected, however morphologically, the *C. olla* accessions varied greatly with respect to basidiocarp characteristics (Fig. 4-1). Basidiocarp diameter ranged from 5-12 mm, while height ranged from 4-11 mm. Most basidiocarps were broad and relatively straight at the mouth (Fig. 4-1a), but others were extremely flared (Fig. 4-1b). The inner peridium wall was generally smooth and grey-tan, however the most striking difference was observed in accession #10, 14, 18, 22, 32, 34, 36, and 40 which had longitudinal ridges on the inner surface of the peridium wall (plicate) (Fig. 4-1c), a distinct characteristic of *C. striatus* and a few other *Cyathus* spp. (Brodie, 1952, 1966, 1967, 1970). The inner surfaces of plicate peridia were typically silver - light brown. The accessions were compared to the DAOM specimens listed in Table 4-2, and most resembled the typical *C. olla* f. *olla*, or *C. olla* f. *anglicus*. No *C. olla* specimens within the DAOM collection were plicate, and the plicate accessions (#10, 14, 18, 22, 32, 34, 36, and 40) did not resemble any of the plicate species (*C. striatus, C. helenae, C. bulleri,* and *C. annulatus)* used for comparison. Basidiocarps that were not in a suitable condition for morphological examination, or were not available for accession #27-31, could not be included in the morphological portion of this study.

Cluster analysis (Fig. 4-2) distinctly grouped the plicate accessions (#10, 14, 18, 32, 34, 36, and 40), and differentiated that cluster from accessions resembling *C. olla* f. *anglicus* (# 4, 8, 35, and 38), and the typical *C. olla* f. *olla*. Accession #37 had many morphological features similar to the plicate group, and therefore, clustered with these accessions even though it lacked the distinctive plicate peridium. The cophenetic correlation was determined to be 0.8580. Morphological examination revealed that accession #6 may actually be *C. stercoreus*, which explains the outlying results obtained for this accession.

4.3.2 Cultural characteristics

When grown on V8 juice rose bengal agar, *C. olla* colonies were typically white.

The marginal areas of the colonies consisted of dense aerial mycelium, but the inner portions formed low growing, cord-like hyphal strands originating from the inoculation plug. After two weeks of growth, accession #19, 23, and 27 developed slight tan pigmentation, and accession #6 failed to produce cords, but appeared sectored and pigmented. Generally, *C. olla* grew slightly faster on PDA, but failed to produce distinct cords. On both of these media, accession #29 and 30 did not produce dense hyphal growth. Colonies of both these accessions were sparse, low growing, and lacked the filamentous and cord-like appearance of all other accessions.

4.3.3 RAPD analysis

Of the 20 primers screened, 13 produced distinct, reproducible RAPD fragments. The number of polymorphic bands produced by each primer varied from 3 to 15, for a total of 110 RAPD markers (Table 4-4). Six of the primers screened did not produce strong amplification products, or did not differentiate between accessions. Only one primer failed to produce any RAPD fragments. RAPD patterns for *C. olla* and *C. striatus* were distinctly different, and the plicate *C. olla* accessions (# 10, 14, 18, 22, 32, 34, 36, and 40) were not similar to *C. striatus* (Figs. 4-3 & 4-4). For these reasons, the statistical analysis and assessment of relatedness was based only on *C. olla*.

The dissimilarity coefficients calculated from the scored data ranged from 0.0 to 0.968. The UPGMA analysis resulted in one tie, affecting the placement of two accessions at a branch point of 0.198. These accessions were contained within a very tightly clustered group, and therefore, did not affect the overall clustering structure of the dendrogram (Fig. 4-5). The cophenetic correlation was determined to be 0.933.

Results from the clustering analysis clearly defined three groups within the collection of *C. olla*. The first cluster consisting of accession #6, 29, and 30 (Group 1, Fig. 4-5) were separated from all other accessions by a genetic distance of 0.936. The other branch contained the majority of the accessions, and further branched into two clusters (Groups 2 and 3) which were separated by a genetic distance of 0.587. The accessions in Group 2 were varied with respect to basidiocarp size, and all but accession #4 and 28 originated from the same geographical area. Accession #10, 14, 18, 32, 34, 36, and 40 (plicate) were also collected from this region but clustered in Group 3.

Although these plicate accessions have clustered in the same group with typical *C. olla* f. *olla* basidiocarp types, they are separated from these types by a genetic distance of 0.403. Accessions #37 and 41 were not plicate, but did cluster with this group. Morphologically, accession #22 appeared faintly plicate, but did not cluster with the distinctly plicate specimens.

4.4 Discussion

Results from the basidiocarp morphology and cultural characteristics indicated much variation among *C. olla*. According to Brodie (1975), the species *C. olla* is described as:

> "Grey or grey-fawn colored fruit bodies usually 10 - 15 mm high, and above all, broad (8 - 10 mm) for the genus; they tend to be markedly expanded or flared outwards near the mouth and mostly thick walled. Externally, the peridium is fine textured, no unevenness or shagginess is apparent to the unaided eye. Internally, the peridium wall is of smooth texture although frequently transversely ridged. The outline of the mouth or lip is commonly wavy and is seldom perfectly circular."

Brodie (1975) noted much variability in size, color, and form, but only recognized three forms, *C. olla* f. *olla*, *C. olla* f. *anglicus*, and *C. olla* f. *lanatus*. *Cyathus olla* f. *anglicus* has a much larger basidiocarp type (up to 15 mm wide x 18 mm high), and is commonly sulcate; and *C. olla* f. *lanatus* is a distinct form from Idaho that has a shaggy peridium, fimbriate lip, wrinkled peridioles, and internal concentric grooves (Brodie, 1978). Variable characters were observed within the collection used in this investigation, however longitudinally ridged, or plicate peridia have not been previously described in *C. olla*. In a morphological key published by Brodie (1977), he firmly states that peridia of *C. olla* are not longitudinally ridged. From this taxonomic description and the morphological groupings of the specimens, it appears that the plicate accessions may be a new form of *C. olla*.

RAPD analysis provided a method to distinguish *C. olla* accessions, and to

assess the degree of variation between them. The resulting dendrogram produced three groups of accessions, two of which were tightly grouped clusters. The most genetically distant group consisted of three accessions that were quite distinct in cultural characteristics from the majority of those in the collection. Since basidiocarps of two of these accessions (#29 and 30) were not available, little comment can be made about their morphology. Generalizations can be made regarding the cluster analysis, but it is clear that the RAPD analysis differentiated the plicate accessions from other basidiocarp types within the collection.

4.4.1 New form

Based on the morphological and RAPD analyses presented, the *C. olla* accessions in this collection can be divided into three types: (i) typical *C. olla*, (ii) larger basidiocarp, and (iii) plicate types, with the first two being morphologically comparable to *C. olla* f. *olla* and *C. olla* f. *anglicus*, respectively. The plicate form was clearly differentiated from these typical *C. olla* forms by both the morphological and RAPD analyses and is described here as a new form.

Cyathus olla (Batch) ex Pers. f. *brodiei* Shinners et Tewari f. nov.

(synonym *Cyathus olla* (Batch) ex Pers. f. *brodiensis* Shinners et Tewari f. nov.)

Forma brodiei differt ab forma olla in eo quod basidiocarpos cum denso sed non hirsuto tomento, amplum sed non attenuatum nec sulcatum os, pallide brunneum sive argenteum interiorem murum peridalem, qui plicatus est, habet; necnon peridiola circiter duorum (2) mm in diametro quae rotunda cum tunica sunt.

Forma *brodiei* differs from the forma *olla* in having basidiocarps with thick but not shaggy tomentum, broad but not flared or sulcate mouth, light brown-silvery distinctly plicated inner peridium wall; peridioles about 2 mm in diameter, round, with a shiny tunica.

HOLOTYPE. CANADA, ALBERTA. Falher, on dead roots of canola (*Brassica napus, B. rapa*), September 16, 1997, T. C. Shinners, deposited in the National Mycological Herbarium of Canada, Ottawa, Ontario as DAOM 225599.

Etymology.--The epithet *brodiei* is in honor of the late Dr. H. J. Brodie of the University of Alberta who dedicated much of his career to the study of the

Nidulariaceae.

This form was originally described and published as *C. olla* f. *brodiensis* by Shinners and Tewari (1998), but this was incorrect as the species epithet indicated a geographical name (Recommendation 60D) and not a personal name (Recommendation 60C.1) (Greuter *et al.*, 1994).

This study determined some of the variability within *C. olla,* but this variation is not expected to be limited to these characteristics only, and is anticipated in all biochemical and ecological processes. This knowledge is imperative for further development of this fungus as a biological control agent. It is not known whether the efficiency of processes such as stubble-colonizing and decomposing capabilities, are linked to the clusters or groupings derived in this study. Further biochemical and ecological research in these areas will provide this information, and help to determine the most effective accession(s) for canola stubble colonization and decomposition.

4.5 Tables and Figures

Table 4-1. *Cyathus olla* accession identification and collection information.

Accession#	and ID	Location	Habitat
1	C94	CANADA, Alberta, Edmonton	agricultural field
2	C95	CANADA, Alberta, Edmonton	agricultural field
3	C96	CANADA, Alberta, Edmonton	agricultural field
4	Rav.	CANADA, Alberta, Edmonton	river valley ravine
5	Beau	CANADA, Alberta, Beaumont	agricultural field
6	IND	USA, Indiana, Indianapolis	agricultural field
7	W240	CANADA, Alberta, Edmonton	agricultural field
8	PR1	CANADA, Alberta, SE 12-79-21-5[a]	agricultural field
9	PR2	CANADA, Alberta, NE 12-82-21-5[a]	agricultural field
10	PR3	CANADA, Alberta, SW 24-78-20-5[a]	agricultural field
11	PR4	CANADA, Alberta, SW 24-78-20-5[a]	agricultural field
12	PR5	CANADA, Alberta, NW 9-78-21-5[a]	agricultural field
13	PR6	CANADA, Alberta, SW 35-79-22-5[a]	agricultural field
14	PR7	CANADA, Alberta, SW 6-80-20-5[a]	agricultural field
15	PR8	CANADA, Alberta, SW 6-80-20-5[a]	agricultural field
16	PR9	CANADA, Alberta, SW 14-78-22-5[a]	agricultural field
17	PR10	CANADA, Alberta, SE 5-78-21-5[a]	agricultural field
18	PR11	CANADA, Alberta, SE 13-79-21-5[a]	agricultural field
19	PR12	CANADA, Alberta, SE 13-79-21-5[a]	agricultural field
20	PR13	CANADA, Alberta, SE 11-78-21-5[a]	agricultural field
21	PR14	CANADA, Alberta, SW 2-79 22-5[a]	agricultural field
22	PR15	CANADA, Alberta, SW 2-79-22-5[a]	agricultural field
23	PR16	CANADA, Alberta, NE 13-81-21-5[a]	agricultural field
24	PR17	CANADA, Alberta, Legal	agricultural field
25	PR18	CANADA, Alberta, NW 16-77-21-5[a]	agricultural field
26	Fair	CANADA, Alberta, Fairview	agricultural field
27	UAMH 8276	UAMH collection[b]	garden
28	DAOM 196679	DAOM collection[c]	unknown
29	DAOM 197563	DAOM collection[c]	unknown

30	DAOM 197577	DAOM collection[c]	unknown
31	DAOM 184718	DAOM collection[c]	unknown
32	PR19	CANADA, Alberta, NW 10-77-21-5[a]	agricultural field
33	PR20	CANADA, Alberta, NW 10-77-21-5[a]	agricultural field
34	PR21	CANADA, Alberta, SE 6-78-20-5[a]	agricultural field
35	PR22	CANADA, Alberta, SE 6-78-20-5[a]	agricultural field
36	PR23	CANADA, Alberta, Falher	agricultural field
37	PR24	CANADA, Alberta, Falher	agricultural field
38	PR25	CANADA, Alberta, NE 33-77-20-5[a]	agricultural field
39	PR26	CANADA, Alberta, NE 33-77-20-5[a]	agricultural field
40	PR27	CANADA, Alberta, SE 26-78-21-5[a]	agricultural field
41	PR28	CANADA, Alberta, SE 26-78-21-5[a]	agricultural field
42	PR29	CANADA, Alberta, NE 20-77-21-5[a]	agricultural field
43	PR30	CANADA, Alberta, NE 20-77-19-5[a]	agricultural field

[a]Legal descriptions within the Municipal District of Smoky River, Alberta, Canada

[b]Obtained from the University of Alberta Microfungus Collection, Edmonton, Alberta, Canada

[c]Obtained from the National Mycological Herbarium of Canada, Ottawa, Ontario, Canada

Table 4-2. *Cyathus* spp. borrowed from the National Mycological Herbarium for study.

Species	Collection number
C. bulleri	DAOM 115667 TYPE, 201161, 201162, 201163, 201164, 201165, 201166, 201167, 201168, 201169, 201170, 201171, 201172, 201173, 201174, 201175, 201176, 201177, 201178, 201179, 201180, 201181, 201182, 201183, 201184
C. helenae	DAOM 200384 TYPE, 200384a TYPE, 200385, 200386, 200387, 200388, 200389, 200390, 200391, 200392, 200393, 200394, 200395, 26409
C. annulatus	DAOM 200366 200367, 200368, 200369
C. striatus	DAOM 25208, 173437, 164790, 152287, 154848, F3302, 206019, 152288, 164790, 206204, 219718
C. olla	DAOM 200786, 200794, 200795, 200796, 200711, 184718, 200733, 200734, 200735, 200736, 200737, 209610, 200727, 200728, 200729, 200730, 200731, 200732, 200738, 200740, 200742, 200739, 200741, 200743, 200744, 200746, 200748, 200745, 200747, 200749, 200750, 200752, 200754, 200751, 200753, 200755, 200756, 200782, 200784, 200781, 200783, 200785
C. olla f. *anglicus*	DAOM 200705, 200706, 200714,
C. olla f. *lanatus*	DAOM 200703 TYPE, 200704 TYPE

Table 4-3. *Cyathus striatus* accession collection information.

Accession #	Location	Habitat
1	USA	garden woodchip mulch
2	USA	garden woodchip mulch
3	USA, Ohio, Chagrin Falls	garden woodchip mulch
4	USA, Ohio, West Lake	garden woodchip mulch
5	USA, Minnesota, St. Paul	garden woodchip mulch
6	CANADA, Alberta, Edmonton	garden woodchip mulch
7	UAMH[a] collection	river bank
8	DAOM[b] collection	unknown
9	DAOM[b] collection	unknown

[a]Obtained from the University of Alberta Microfungus Collection, Edmonton, Alberta

[b]Obtained from the National Mycological Herbarium of Canada, Ottawa, Ontario, Canada

Table 4-4. Primers used for RAPD analysis

Primer	Sequence (5' to 3')	# of RAPD markers produced
OPA-01	CAGGCCCTTG	8
OPA-02	TGCCGAGCTG	5
OPA-03	AGTCAGCCAC	15
OPA-04	AATCGGGCTG	10
OPA-05	AGGGGTCTTG	3
OPA-07	GAAACGGGTG	12
OPA-08	GTGACGTAGG	8
OPA-09	GGGTAACGCC	7
OPA-10	GTGATCGCAG	9
OPA-13	CAGCACCCAC	10
OPA-16	AGCCAGCGAA	7
OPA-18	AGGTGACCGT	8
OPA-20	GTTGCGATCC	8
Total		110

Figure 4-1. Basidiocarp morphologies of *Cyathus olla*. (5x)

(a) Typical *C. olla* f. *olla*.

(b) Type with larger basidiocarp, *C. olla* f. *anglicus*.

(c) Type with plicate peridium, *C. olla* f. *brodiei* f. nov.

Figure 4-2. Dendrogram of *Cyathus olla* accessions based on 12 morphological characters and generated by UPGMA clustering analysis using NTSYS-pc. The cophenetic correlation was determined to be 0.8580. Accession numbers correspond to those in Table 4-1.

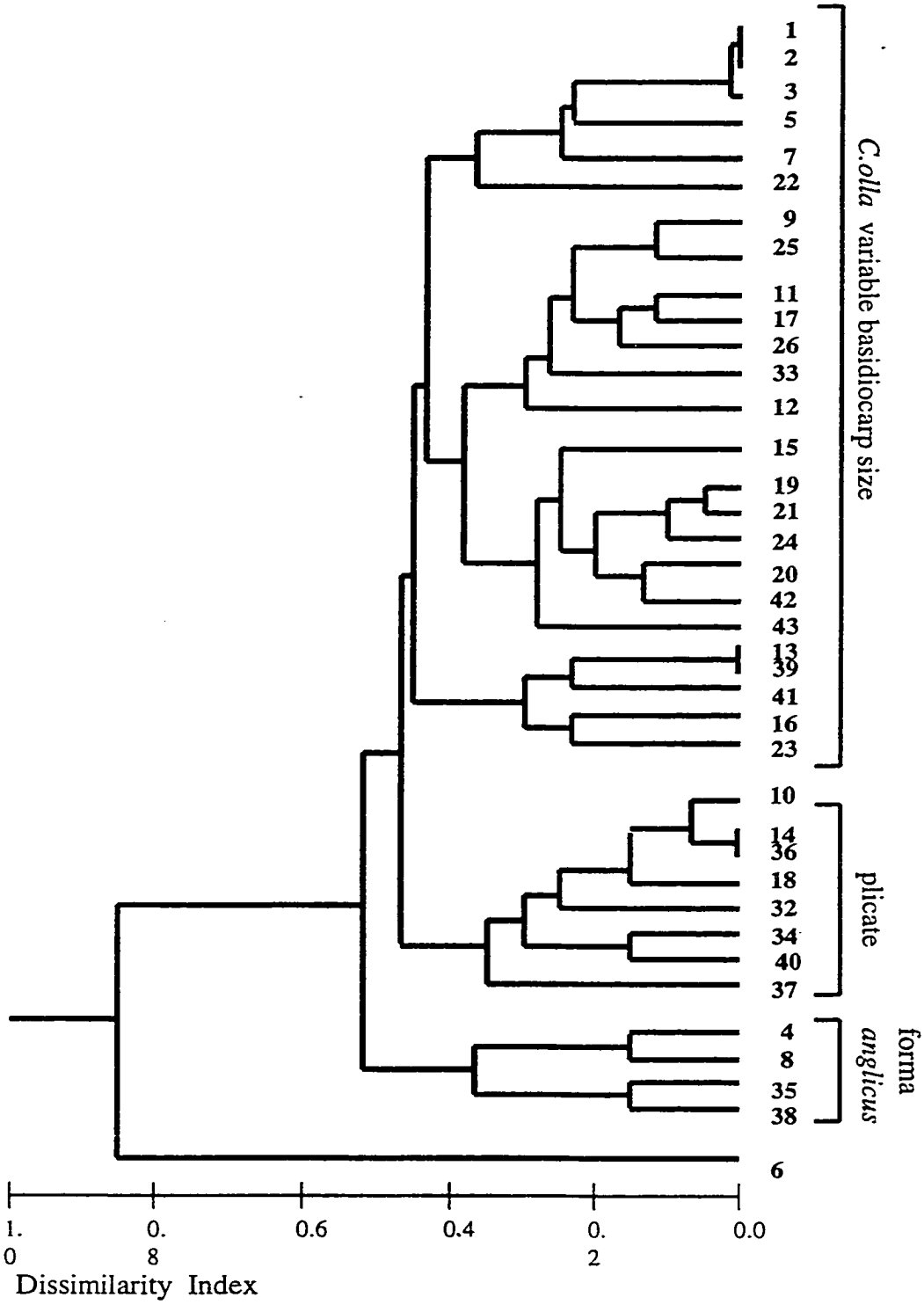
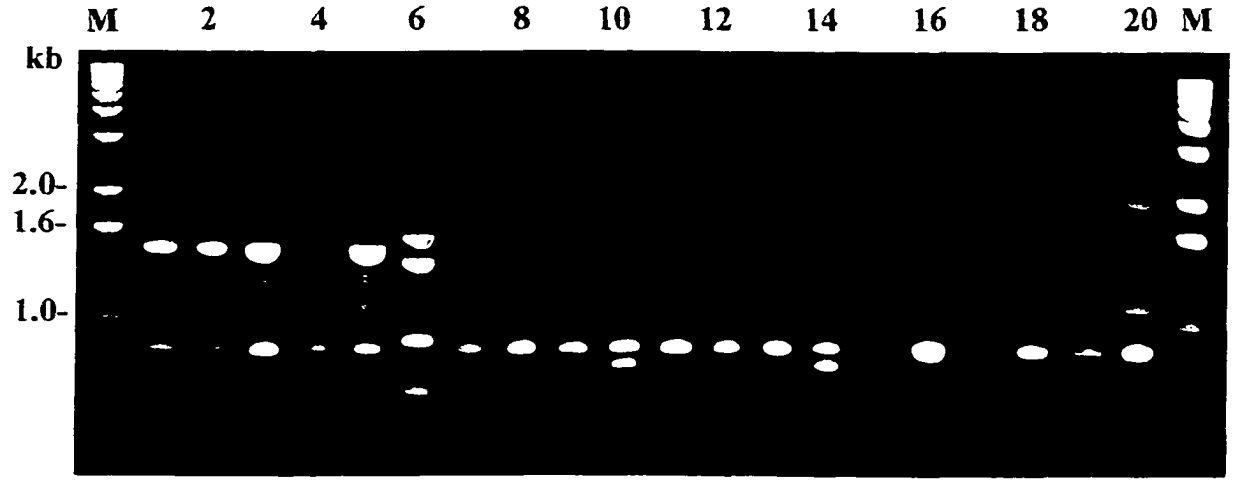
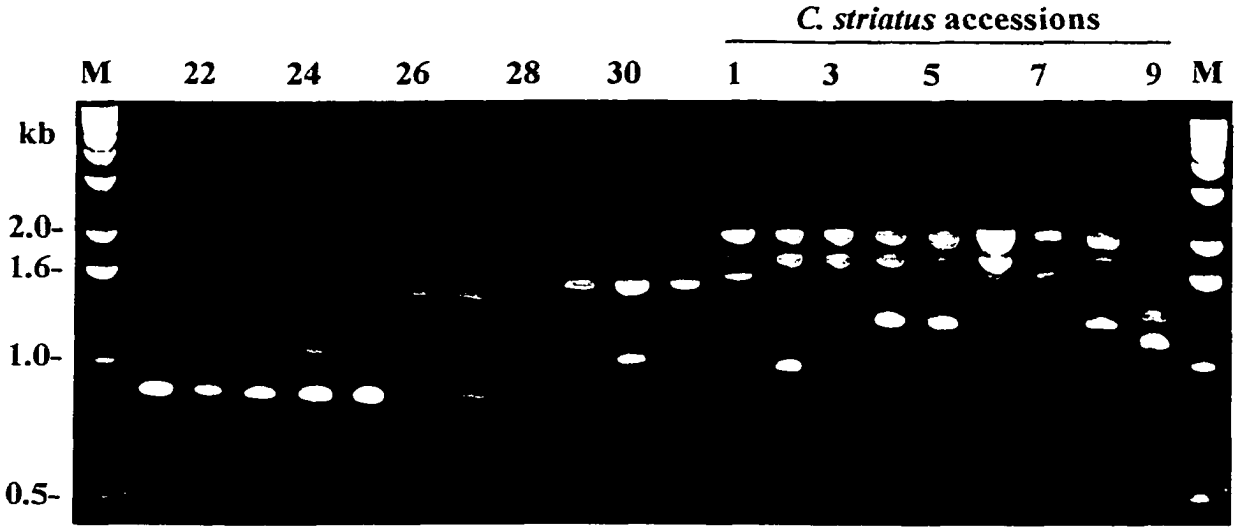


Figure 4-3. RAPD patterns of 43 *Cyathus olla* and 9 *Cyathus striatus* accessions obtained by amplification of total DNA using 10-mer primer, OPA02 (5'-TGCCGAGCTG- 3'). Lane numbers correspond to accession numbers in Tables 4-1 and 4-2. DNA ladder (1kb) indicated as M.





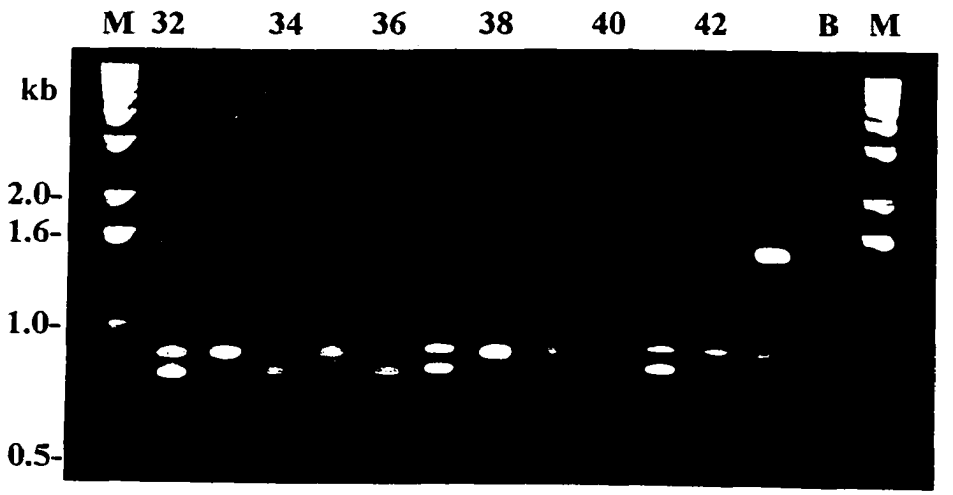
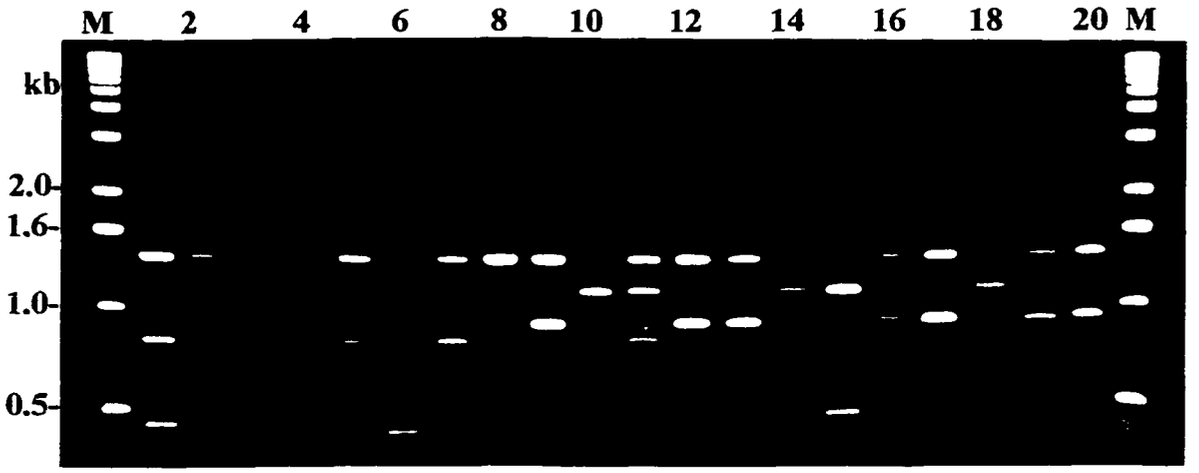
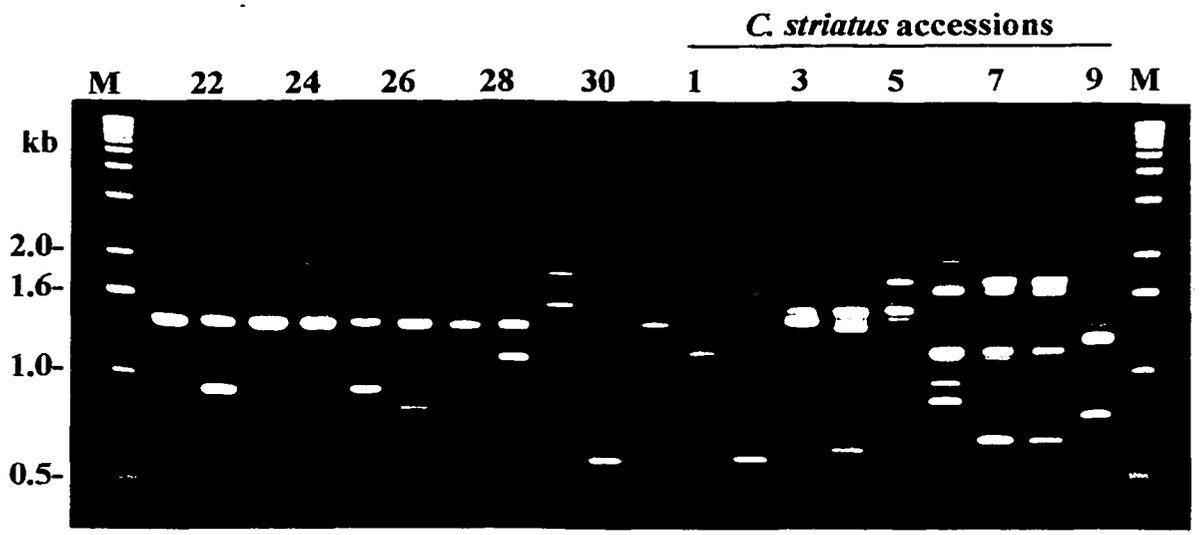


Figure 4-4. RAPD patterns of 43 *Cyathus olla* and 9 *Cyathus striatus* accessions obtained by amplification of total DNA using 10-mer primer, OPA20 (5'-GTTGCGATCC- 3'). Lane numbers correspond to accession numbers in Tables 4-1 and 4-2. DNA ladder (1kb) indicated as M.





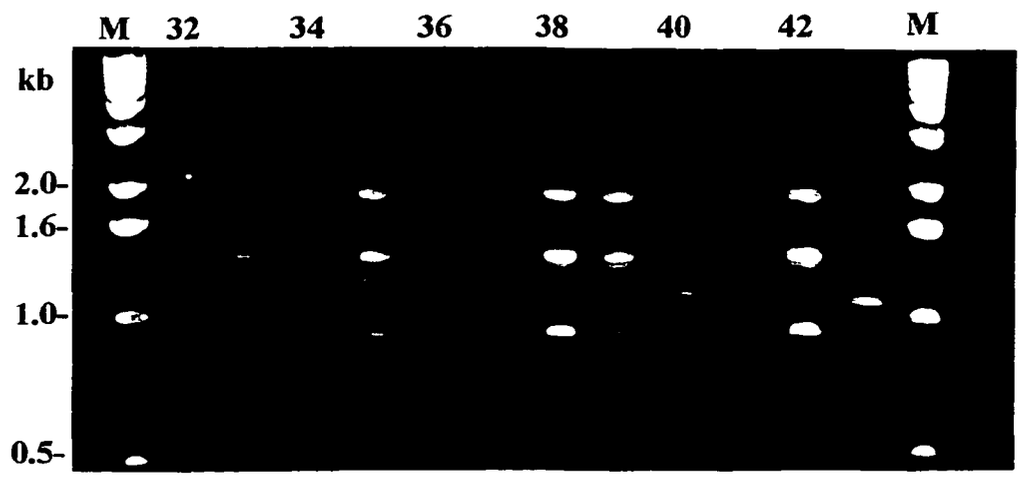
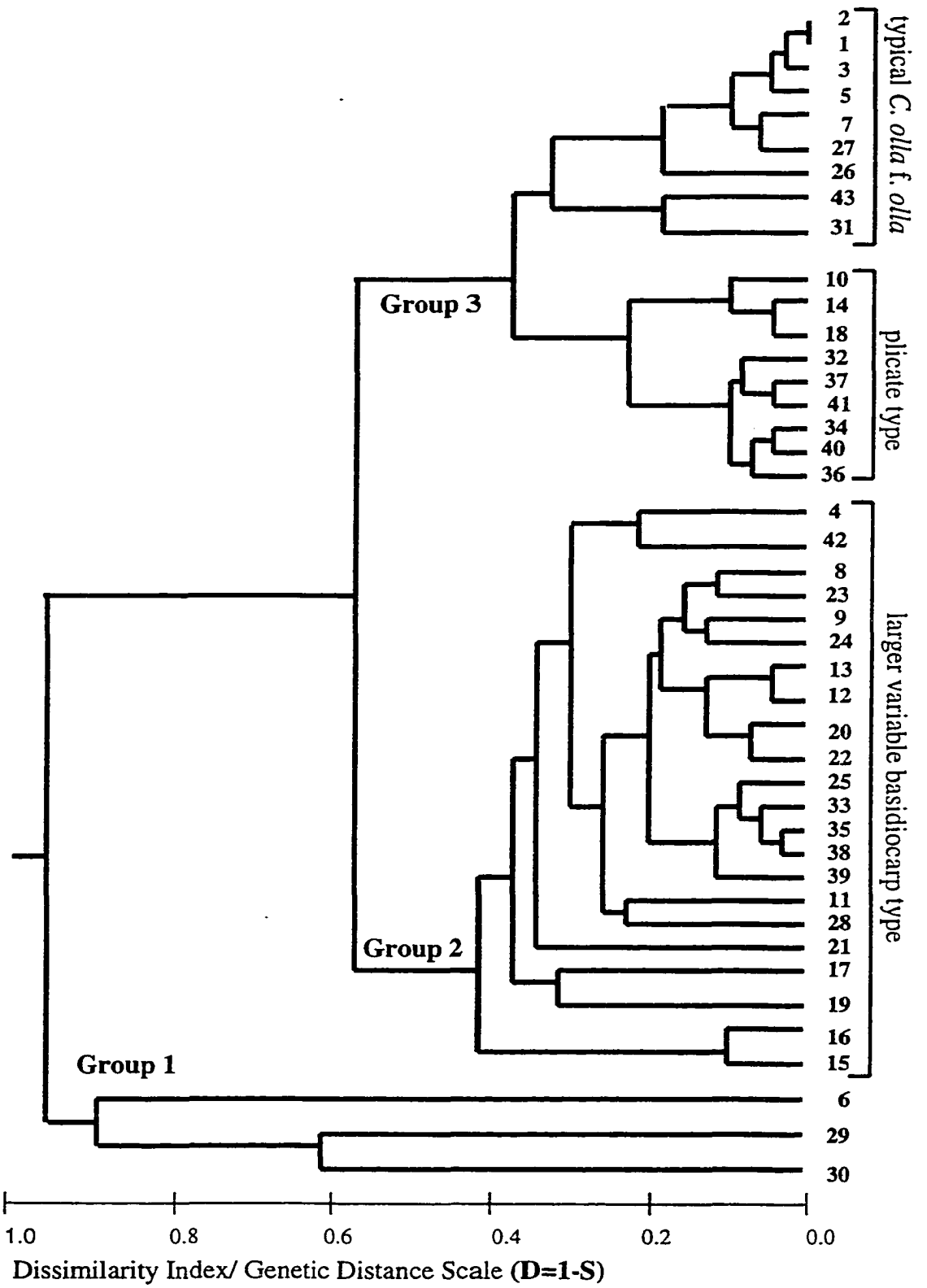


Figure 4-5. Dendrogram of 43 *Cyathus olla* accessions based on genetic distances/ dissimilarity values from RAPD analyses and generated by UPGMA clustering analysis using NTSYS-pc. The cophenetic correlation was determined to be 0.933. Accession numbers correspond to those in Table 4-1.



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Chapter 5

Enzymatic activity of *Cyathus olla* during solid state fermentation of canola root material. [1]

5.1 Introduction

As white wood-rotting fungi, *Cyathus* spp. are ecologically specialized to degrade lignocellulosic material (Abbott and Wicklow, 1984; Wicklow *et al.*, 1984), but the enzymes responsible for lignocellulosic metabolism by these fungi are not well known. Manganese peroxidase (MnP) and laccase have been identified in *C. stercoreus* (Orth *et al.*, 1993), and cellulases have been detected in *C. helenae* (Kuhad and Johri, 1991; Kuhad and Johari, 1987), *C. striatus,* and *Cyathus* sp. (Kuhad and Johari, 1987). However little information exists with respect to the plant cell wall degrading enzymes produced by *C. olla.* In a screening experiment, Pelaez *et al.* (1995) detected aryl-alcohol oxidase (AAO) from *C. olla,* but did not detect any other ligninolytic enzymes.

In order to investigate the proposed use of *C. olla* as a biological control agent, the enzymatic potential of this fungus must be understood. Scanning electron microscopy of field collected canola stubble infested with *C. olla* revealed the presence of calcium oxalate crystals and structural deterioration of the substrate, indicative of enzymatic activity and subsequent decomposition (Tewari *et al.*, 1997; Chapter 2). From these results, it is assumed that *C. olla* is actively involved in the degradation process, but in order to confirm this, the growth of *C. olla* on a canola substrate must be studied, and an attempt made to detect a degradative enzyme system. For this reason, the objectives of this study were to detect and identify plant cell wall degrading enzymes produced by *C. olla* during solid state fermentation of canola root material. Considering the substrate specialization of this fungus, emphasis was placed on detecting the ligninolytic enzyme system, and identifying the individual enzymes involved. Cellulase

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[1] *A version of this chapter has been submitted for publication:* Shinners-Carnelley, T.C.; A. Szpacenko; J.P. Tewari; and M.M. Palcic. Applied and Environmental Microbiology (submitted)

and polygalacturonase activities were tested, but were not the main focus of this research.

5.2 Materials and Methods

5.2.1 Detection of ligninolytic accessions

As a screening procedure, 42 *C. olla* accessions including three forms of the species (*C. olla* f. *olla*, *C. olla* f. *anglicus*, and *C. olla* f. *brodiei*) were tested for the ability to degrade a model lignin substrate, the polymeric dye Poly R-478 (Sigma) (Glenn and Gold, 1983). Accessions were collected from northern and central Alberta, Canada, and obtained from the University of Alberta Microfungus Collection, Edmonton, Alberta, Canada, and the National Mycological Herbarium of Canada, Ottawa, Ontario, Canada (Table 5-1). *Phanerochaete chrysosporium* (UAMH 3642) was used as a positive lignin degrading control. An uninoculated plate was used as a negative control. All isolates were sub-cultured from potato dextrose agar onto a medium containing KH_2PO_4, 0.6g/ L; $MgSO_4$. $7H_2O$, 0.5g/ L; K_2HPO_4, 0.4g/ L; $(NH_4)_2$ tartrate, 0.22g/ L; sorbose, 40.0 g/ L; Poly R-478 dye (Sigma), 0.2 g/ L; agar, 15.0 g/ L; mineral solution ($CaCl_2$. $2H_2O$, 7.4 g/ L; ferric citrate, 1.2 g/ L; $ZnSO_4$. $7H_2O$, 0.7 g/ L; $MnSO_4$. $4H_2O$, 0.5 g/ L; $CoCl_2$. $6H_2O$, 0.1 g/ L; thiamine HCl, 10.0 mg/ L), 10.0 mL (Paterson and Bridge, 1994) and maintained at 25°C in the dark. Culture plates were observed weekly, and zones of decolorization were measured and recorded after 4-wks (Table 5-1).

Based on these results, 10 *C. olla* accessions and *P. chrysosporium* were selected for spectrophotometrically testing of their ability to degrade the polymeric dye in a liquid medium containing canola root powder, 2.0 g/ L, in 10 mM succinic acid dimethyl ester, pH 4.5, and Poly R-478, 0.2 g/ L. Sterile media were dispensed into 75 mL sterile screw cap tissue culture flasks and inoculated with an agar plug. Following inoculation, all cultures were aerated with oxygen for 10 min at a flow rate of 100-150 mL/ min, and daily thereafter for 2 wks. Cultures were maintained under the same conditions as described above. Each day for 2 wks, aliquots of filtrate were removed and diluted 10 fold with water. Decolorization was determined by measuring the

change in absorbance ratio (A_{520}/ A_{350}) (De Jong *et al.*, 1992). After 4 wks, the final absorbance ratio was determined. All accessions were cultured and measured in triplicate.

5.2.2 Preliminary identification of ligninolytic enzymes from culture filtrate

In an attempt to identify the enzymes responsible for decolorization of the polymeric dye, five *C. olla* accessions were grown under the conditions described above, with the exception that the medium lacked Poly R-478. After 1 wk of growth, the culture fluid was separated by centrifugation at 15000 x *g* at 4°C and assayed for ligninolytic enzymes. This experiment was also repeated without daily flushing of O_2 in order to determine the effectiveness of this step.

5.2.3 Ligninolytic enzyme assays

All assays were carried out in 1 mL volumes at room temperature and the absorbance was measured using a Hewlett Packard 8451A diode array spectrophotometer. One unit of activity was defined as the amount of enzyme required to produce 1 µmol of product per min (1U= 1µmol/ min, 1mU= 1nmol/ min).

Lignin peroxidase (LiP) was measured by the oxidation of veratryl alcohol to veratrylaldehyde (E_{310}= 9300 $M^{-1}cm^{-1}$) (Tien and Kirk, 1988). Each assay contained 50 mM sodium tartrate pH 2.5, 2 mM veratryl alcohol, and 10-550 µL enzyme solution. The reaction was initiated with the addition of 0.4 mM H_2O_2. Managanese peroxidase was assayed according to Paszczynski *et al.* (1988) using 0.01% phenol red as the substrate (E_{610}= 4460 $M^{-1}cm^{-1}$). Each assay contained 100 mM sodium tartrate pH 5.0, 0.01% (w/v) phenol red, 0.1 mM $MnSO_4$, and 10-690 µL enzyme solution. The reaction was initiated with 0.1 mM H_2O_2. Laccase activity was measured by the oxidation of 2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonate) (ABTS) (E_{420}= 3.6 x 10^4 M^{-1} cm^{-1}) (Bourbonnais and Paice, 1990). Each assay contained 0.5 mM ABTS, 0.1 M sodium acetate buffer pH 5.0, and 10-700 µL of enzyme solution. Aryl-alcohol oxidase was assayed according to Guillen *et al.* (1992) by measuring the oxidation of veratryl alcohol to veratrylaldehyde (E_{310}= 9300 M^{-1} cm^{-1}). Each assay contained 100 mM sodium

phosphate buffer pH 6.0, 10 mM veratryl alcohol and up to 550 µL of enzyme solution. Prior to performing the assays, veratryl alcohol was purified to prevent trace impurities from interfering with the assays (Tien and Kirk, 1988).

5.2.4 Solid state fermentation of *C. olla* on canola root material

One *C. olla* isolate (PR1) was selected for solid state fermentation of canola root material. Incubations were carried out in 1L Erlenmeyer flasks that contained 10g of canola root material (ground in a Wiley mill, 1mm screen) and 40 mL of distilled H_2O. The flasks were sterilized three times at 121°C for 35 min. Following the first sterilization, an additional 10 mL of distilled H_2O was added. Sterile flasks were inoculated with 10 mL of the mycelial suspension, and incubated at 25°C in the dark for 1- or 4-wks.

The contents of two flasks were harvested for each enzyme preparation. Colonized root material was suspended in 300 mL of cold 25 mM sodium acetate buffer, pH 5.5, and sonicated for two 30 second periods, followed by gentle shaking for 30 min at room temperature. This procedure was repeated twice, for a total of six 30 sec. sonication cycles and 1.5 hours of shaking. The suspension was centrifuged at 25931 x *g* for 30 min at 4°C. The supernatant was removed, and each pellet was washed with 50 mL of 25 mM sodium acetate buffer, pH 5.5 and re-centrifuged as above. Following this step, the supernatant (approximately 400 mL) was filtered through Whatman #1 filter paper to clarify the extract. Aliquots were removed to initially assay the enzyme activities including LiP, MnP, laccase, and AAO. Two 20 mL aliquots were also removed and lyophilized from each preparation for total cellulase and polygalacturonase assays.

5.2.5 Protein purification

The proteins contained in the extract were fractionated by anion-exchange chromatography on Q-Sepharose Fast Flow medium (Pharmacia). The column (2.6 x 18 cm) was equilibrated with 25 mM sodium acetate, pH 5.5 and approximately 360 mL of the extract was loaded, followed by an additional wash with 1 L, 25 mM sodium

acetate, pH 5.5. Proteins were eluted using a linear NaCl gradient of 0 to 0.3 M, with a total elution volume of 500 mL. Following this, the column was washed with 1M NaCl. The flow rate was 1.6 mL/ min, and the eluted proteins were collected in 3.7 mL fractions. Enzyme activities (assays previously described) and heme (408 nm) were measured as the fractions were collected. Fractions with activity were pooled and concentrated using Slide-a-Lyzer cassettes (Pierce). Protein determinations (Bradford, 1976) were made at each step using the Coomassie Plus (Pierce) micro-plate assay.

This extraction and purification procedure was adapted from a method of Vares *et al.* (1995) that was used to extract and identify ligninolytic enzymes from wheat straw inoculated with *Phlebia radiata*.

5.2.6 Cellulases

Lyophilized samples were re-dissolved in 2 mL (one-tenth their original volume) of 50 mM citrate buffer, pH 4.8 and dialyzed against the same buffer. Total cellulase activity was determined using the filter paper method (Mandels *et al.*, 1976) and the dinitrosalicyclic acid (DNS) method for determination of reducing sugars (Miller *et al.*, 1960). Enzyme and substrate blanks were included to measure the background levels of reducing sugars. A glucose standard curve (0-4 mg) was used to calculate enzyme activity.

5.2.7 Polygalacturonase

Lyophilized samples were re-dissolved in 2 mL (one-tenth their original volume) of 50 mM sodium acetate buffer, pH 5.2 and dialyzed against the same buffer. Polygalacturonase activity was determined by the release of reducing sugars from 0.1% (w/v) polygalacturonic acid. Each assay contained 5-20 µL of the concentrated enzyme preparation in 0.8 mL of 50 mM sodium acetate buffer, pH 5.2 with 0.1% polygalacturonic acid, and adjusted to 1 mL with buffer (Annis and Goodwin, 1997). The samples were incubated for 1h at 30°C and reducing sugars were measured using the DNS method. A galacturonic acid standard curve (0-0.2 mg) was used to calculate polygalacturonase activity.

5.3 Results

5.3.1 Detection of ligninolytic activity

Of the 42 accessions screened, all but two (DAOM 197563 and DAOM 197577) decolorized the Poly R-478 medium to some degree over the 4-wk period. Zone of decolorization was determined by measuring the diameter of the decolorized area (Table 5-1). *Phanerochaete chrysosporium* decolorized the entire plate (diameter 90 mm), whereas 40 accessions of *C. olla* produced decolorized zones ranging in diameter from 20-59 mm (Fig. 5-1, Table 5-1).

The 10 accessions that produced the largest zones of decolorization were selected for the spectrophotometric analysis. Decolorization by *C. olla* was initiated within 3 d following inoculation, and appeared to be maximized at day 8 (192 h post-inoculation) (Fig. 5-2). Over the initial 2-wk period, all *C. olla* accessions decolorized the dye to a greater extent than *P. chrysosporium*. However after 4 wks, the absorbance ratio of 0.210 for this species was significantly lower than that of the *C. olla* accessions which had a mean absorbance ratio of 0.237. There was no significant difference between the 10 *C. olla* accessions with respect to decolorization of Poly R-478 after 4-wks incubation.

The five accessions used for the preliminary ligninolytic enzyme assays included PR1, PR5, PR6, PR10, and PR13. These were chosen randomly from the accessions used in the spectrophotometric assay since there were no significant differences observed. Assays of the crude filtrate detected laccase from all of the *C. olla* accessions, but not from *P. chrysosporium*. Accessions that received daily flushes of O_2 produced 1.41 mU/ mL of laccase activity, which was significantly lower ($p=0.05$) than 4.52 mU/ mL of laccase activity produced by these accessions when they were not flushed with O_2. Laccase production among the chosen accessions was not significantly different ($p=0.05$). No other ligninolytic enzymes were detected in the *C. olla* accessions. The preliminary screening enzyme assays of crude culture filtrate detected only one enzyme, suggesting that enzyme isolation, purification, and concentration would be necessary to study the ligninolytic enzyme system of *C. olla*.

5.3.2 Detection of ligninolytic enzymes from solid-state fermentation of canola

Accession PR1, *C. olla* f. *anglicus,* was chosen for the solid-state fermentation experiment. Only one accession was chosen due the limitations of this experiment. Initial buffer extract samples from both the 1-wk and 4-wk preparations were assayed for LiP, MnP, laccase, and AAO. Laccase and MnP activities were detected from both incubation periods (Table 5-2), but LiP and AAO were not detected in the initial crude extract.

Buffer extracts from both the 1-wk and 4-wk incubations were brown in color. When loaded in the column, a brown zone appeared bound to the Q-Sepharose that was not removed with the buffer wash. This zone remained tightly bound through the NaCl gradient, and was not eluted until the column was washed with 1M NaCl. As fractions were eluted, they were assayed for ligninolytic enzyme activity, and heme was measured as an indicator for the heme containing enzymes, LiP and MnP. Laccase activity was the first enzyme to be detected in the elution profiles in both 1-wk (Fig. 5-3) and 4-wk (Fig. 5-4) incubations. Increase in heme absorbance was useful in detecting MnP, which was èluted from the column soon after laccase, with a few fractions containing both enzymes. After 1-wk, there were two peaks of MnP activity (Fig. 5-3)suggesting two forms of this enzyme, but after a 4-wk incubation period, only one MnP peak was discernible (Fig. 5-4). Aryl alcohol oxidase was not detected in enzyme assays prior to fractionation, but two peaks of enzyme activity were observed in a narrow range of the elution profile from the 4-wk preparation. Lignin peroxidase was not detected throughout the experiment.

Eluted fractions with activity were pooled and concentrated using the Slide-a-Lyzer cassette technique (Pierce). Enzyme and protein assays of these concentrated fractions were used to determine the specific activity of each enzyme (Table 5-3). Specific activity values increased as a result of anion-exchange chromatography on Sepharose-Q Fast Flow medium and concentration techniques, demonstrating that these methods were successful at separating, and to some degree, purifying the ligninolytic enzymes present. Replicate experiments of these incubation periods and extractions

produced reproducible elution profiles, whereby, the enzymes were eluted at the same place in the gradient. However, the activities of each enzyme were variable between replicate incubations. Laccase was the most abundant enzyme produced. Initial extracts from 1-wk incubations ranged from approximately 12-27 U of total activity, but consistently produced approximately 20 U of activity in the 4-wk incubations. Manganese peroxidase from the initial extract was more variable, ranging from being not detectable to approximately 1.3 U of total activity from both incubation periods. Detection of AAO was confounded by the apparent instability of the enzyme. Aryl alcohol oxidase activity decreased sharply within 24 h following fractionation, but this was not a problem with laccase or MnP.

5.3.3 Cellulase and polygalacturonase

Using the filter paper method, no cellulase activity was detected from either the 1-wk or 4-wk incubation preparations. Assays of substrate blanks determined that the enzyme solution had a high background of reducing sugars, with the 1-wk and 4-wk incubation preparations having 2.03 and 2.19 mg of reducing sugar/ mL of enzyme solution, respectively. Polygalacturonase activity was detected from both the 1-wk and 4-wk incubation preparations (186.6 and 60.3 mU/ mg protein respectively) (Table 5-2).

5.4 Discussion

The preliminary assays using Poly R-478 decolorization provided a relatively simple, time efficient, and inexpensive method for the detection of the ligninolytic enzyme system and selection of accessions for further experiments. No significant differences in decolorization or laccase activity were observed among accessions under the conditions used in this experiment, however O_2 concentration did affect enzyme activity, whereby increased O_2 levels resulted in lower laccase activity. *In vitro* activation of the ligninolytic enzyme system of white-rot fungi is generally associated with the presence of oxygen. This enzyme system is complex, and O_2 is thought to be involved in degradative reactions and production of H_2O_2, which is required by the peroxidase enzymes (Kirk and Farrell, 1987). However, Reid and Seifert (1982) have

demonstrated that several species of white rot fungi differed in their response to an elevated O_2 atmosphere.

The results presented here demonstrate that *C. olla* has a ligninolytic enzyme system that is active during solid state fermentation in a canola substrate. This appears to be the first report of laccase and MnP production by this fungus. Both of these enzymes were detected after 1-wk and 4-wk incubation periods. Aryl-alcohol oxidase was not initially detected, but was present after 4-wks of growth. These results are similar to those of Pelaez *et al.* (1995) who did not detect this enzyme from *C. olla* after 7 d of growth, but did find AAO in the culture medium following 21 d incubation. As cultures age, enzyme profiles change. Vares *et al.* (1995) observed such changes at weekly intervals over a 4-wk period of growth of *Phlebia radiata* on wheat straw.

From Fig. 5-4, it appears that laccase activity decreased, and MnP activity increased by the fourth week of incubation, relative to the 1-wk preparations. This could be a valid trend, but in this situation, it cannot be assumed that the differences in laccase and MnP activities are significant since enzyme activity was variable between replicate incubation periods. These enzymes were produced in all replicates, and were eluted at the same places in the NaCl gradient, but their relative activities were variable from preparation to preparation. The canola root material was used as a substrate based on practical considerations relative to the objectives of this experiment, but was perhaps not as well defined as an artificial medium, and therefore, may have been a possible reason for the variability observed in these experiments. The optimum conditions required for the ligninolytic activity of *C. olla* have not been defined, but when determined could result in more consistency with respect to enzyme activity. Further research should be conducted to elucidate these conditions, but for the purpose of this experiment, the results were significant and contributed to understanding the role of *C. olla* in canola stubble decomposition. Previous studies revealed that this fungus produces calcium oxalate crystals (Tewari *et al.*, 1997; Chapter 2), suggesting oxalic acid secretion and calcium sequestration. The results presented provide further information regarding these activities by confirming the production of plant cell wall

degrading enzymes that are thought to be active following calcium sequestration, and subsequent weakening of the substrate (Bateman and Beer, 1965).

No cellulases were detected under these cultivation conditions. Cellulase enzymes are induced by low glucose or sugar concentrations, and are repressed when these sugars are in excess of fungal requirements. A high background level of reducing sugars was present, and might have contributed to a repressive effect under these conditions. The canola substrate used might have had sufficient soluble sugars to fulfill the carbohydrate requirement of the fungus throughout the time course of this experiment. Polygalacturonase activity was detected from both incubation periods. There are very few reports in the literature of pectinase enzymes associated with white rot fungi. The fungi in this group are specialized to degrade cellulose and lignin, and the assumption is often made that it is not ecologically significant for white-rot fungi to degrade pectin, since it makes up only a small fraction of wood. However after detecting polygalacturonase from brown-rot fungi, Green *et al.* (1995) suggested that the importance of pectinase enzymes in wood decay may be underestimated. The ability of a wood-rotting fungus to hydrolyse pectic substances may be advantageous, resulting in solubilization of bordered pit membranes, allowing access to adjoining tracheids. Therefore, the location of pectic substances in woody tissues may be more significant than the quantity.

The ability of *C. olla* to produce polygalacturonase is a significant finding, but it is not known whether this ability would be induced in a field situation within the time intervals used in this experiment. Stubble decomposition is a complex process, wherein many ecologically specialized microorganisms and soil fauna are involved in natural succession on the substrate. From field observations, *C. olla* is normally found growing on decorticated stubble. In the natural succession of organisms, it is assumed that the first to colonize and decorticate stubble would be microorganisms most successful at competing for, and utilizing the soluble sugars, pectic substances, and other easily degraded components. *Cyathus olla* would colonize after these other saprophytes could no longer obtain nutrients from the remaining lignocellulose. At this point,

polygalacturonase production by *C. olla* may be significant, and aid in colonization of the substrate, as suggested by Green *et al.* (1995).

The results presented here have identified that *C. olla* is capable of producing laccase, MnP, AAO, and polygalacturonase during solid state fermentation of canola root material. These findings contribute to the growing body of knowledge on biochemical and ecological attributes of this fungus that is required to assess its potential of being developed into a microbial inoculant to accelerate stubble decomposition, and ultimately to reduce the incidence of stubble-borne diseases of canola. This innovative application would be beneficial to the agricultural industry, but *C. olla* may also prove to be suited to other applications requiring degradation of aromatic rings such as soil remediation, pesticide degradation, or delignification of other lignocellulosic substrates.

5.5 Tables and Figures

Table 5-1. *Cyathus olla* accession-information and results of the Poly R-478 plate assay.

ID	Form	Location	Diameter of decolorized zone (mm)
C94	*C. olla* f. *olla*	CANADA, Alberta, Edmonton	39
C95	*C. olla* f. *olla*	CANADA, Alberta, Edmonton	32
C96	*C. olla* f. *olla*	CANADA, Alberta, Edmonton	37
Rav.	*C. olla* f. *anglicus*	CANADA, Alberta, Edmonton	28
Beau	*C. olla* f. *olla*	CANADA, Alberta, Beaumont	46
W240	*C. olla* f. *olla*	CANADA, Alberta, Edmonton	20
PR1	*C. olla* f. *anglicus*	CANADA, Alberta, SE 12-79-21-5[a]	51
PR2	*C. olla* f. *olla*	CANADA, Alberta, NE 12-82-21-5[a]	20
PR3	*C. olla* f. *brodiei*	CANADA, Alberta, SW 24-78-20-5[a]	32
PR4	*C. olla* f. *olla*	CANADA, Alberta, SW 24-78-20-5[a]	34
PR5	*C. olla* f. *olla*	CANADA, Alberta, NW 9-78-21-5[a]	47
PR6	*C. olla* f. *olla*	CANADA, Alberta, SW 35-79-22-5[a]	59
PR7	*C. olla* f. *brodiei*	CANADA, Alberta, SW 6-80-20-5[a]	34
PR8	*C. olla* f. *olla*	CANADA, Alberta, SW 6-80-20-5[a]	38
PR9	*C. olla* f. *olla*	CANADA, Alberta, SW 14-78-22-5[a]	40
PR10	*C. olla* f. *olla*	CANADA, Alberta, SE 5-78-21-5[a]	50
PR11	*C. olla* f. *brodiei*	CANADA, Alberta, SE 13-79-21-5[a]	25
PR12	*C. olla* f. *olla*	CANADA, Alberta, SE 13-79-21-5[a]	21
PR13	*C. olla* f. *olla*	CANADA, Alberta, SE 11-78-21-5[a]	40
PR14	*C. olla* f. *olla*	CANADA, Alberta, SW 2-79 22-5[a]	41
PR15	*C. olla* f. *olla*	CANADA, Alberta, SW 2-79-22-5[a]	49
PR16	*C. olla* f. *olla*	CANADA, Alberta, NE 13-81-21-5[a]	39
PR17	*C. olla* f. *olla*	CANADA, Alberta, Legal	45
PR18	*C. olla* f. *anglicus*	CANADA, Alberta, NW 16-77-21-5[a]	37
PR19	*C. olla* f. *brodiei*	CANADA, Alberta, NW 10-77-21-5[a]	25
PR20	*C. olla* f. *olla*	CANADA, Alberta, NW 10-77-21-5[a]	24

PR21	*C. olla* f. *brodiei*	CANADA, Alberta, SE 6-78-20-5[a]	20
PR22	*C. olla* f. *anglicus*	CANADA, Alberta, SE 6-78-20-5[a]	47
PR23	*C. olla* f. *brodiei*	CANADA, Alberta, Falher	30
PR24	*C. olla* f. *olla*	CANADA, Alberta, Falher	20
PR25	*C. olla* f. *anglicus*	CANADA, Alberta, NE 33-77-20-5[a]	28
PR26	*C. olla* f. *olla*	CANADA, Alberta, NE 33-77-20-5[a]	40
PR27	*C. olla* f. *brodiei*	CANADA, Alberta, SE 26-78-21-5[a]	30
PR28	*C. olla* f. *olla*	CANADA, Alberta, SE 26-78-21-5[a]	30
PR29	*C. olla* f. *olla*	CANADA, Alberta, NE 20-77-21-5[a]	45
PR30	*C. olla* f. *olla*	CANADA, Alberta, NE 20-77-19-5[a]	37
Fair	*C. olla* f. *olla*	CANADA, Alberta, Fairview	45
UAMH 8276	*C. olla* f. *olla*	UAMH collection[b]	49
DAOM 196679	*C. olla* f. *olla*	DAOM collection[c]	20
DAOM 197563	*C. olla* f. *olla*	DAOM collection[c]	0
DAOM 197577	*C. olla* f. *olla*	DAOM collection[c]	0
DAOM 184718	*C. olla* f. *olla*	DAOM collection[c]	25

[a]Legal descriptions within the Municipal District of Smoky River, Alberta, Canada

[b]Obtained from the University of Alberta Microfungus Collection, Edmonton, Alberta, Canada

[c]Obtained from the National Mycological Herbarium of Canada, Ottawa, Ontario, Canada

Table 5-2. Initial enzyme activities in culture extracts from canola root degraded by *Cyathus olla* PR1.

	Specific Activity (mU/ mg protein)*	
Enzyme	**1 week**	**4 week**
Laccase	923	300
Mn P	18	21
AAO	ND	ND
Total cellulases	ND	ND
Polygalacturonase	187	60

ND- Not detected

*1 mU = 1 nmol of product/ min/ ml

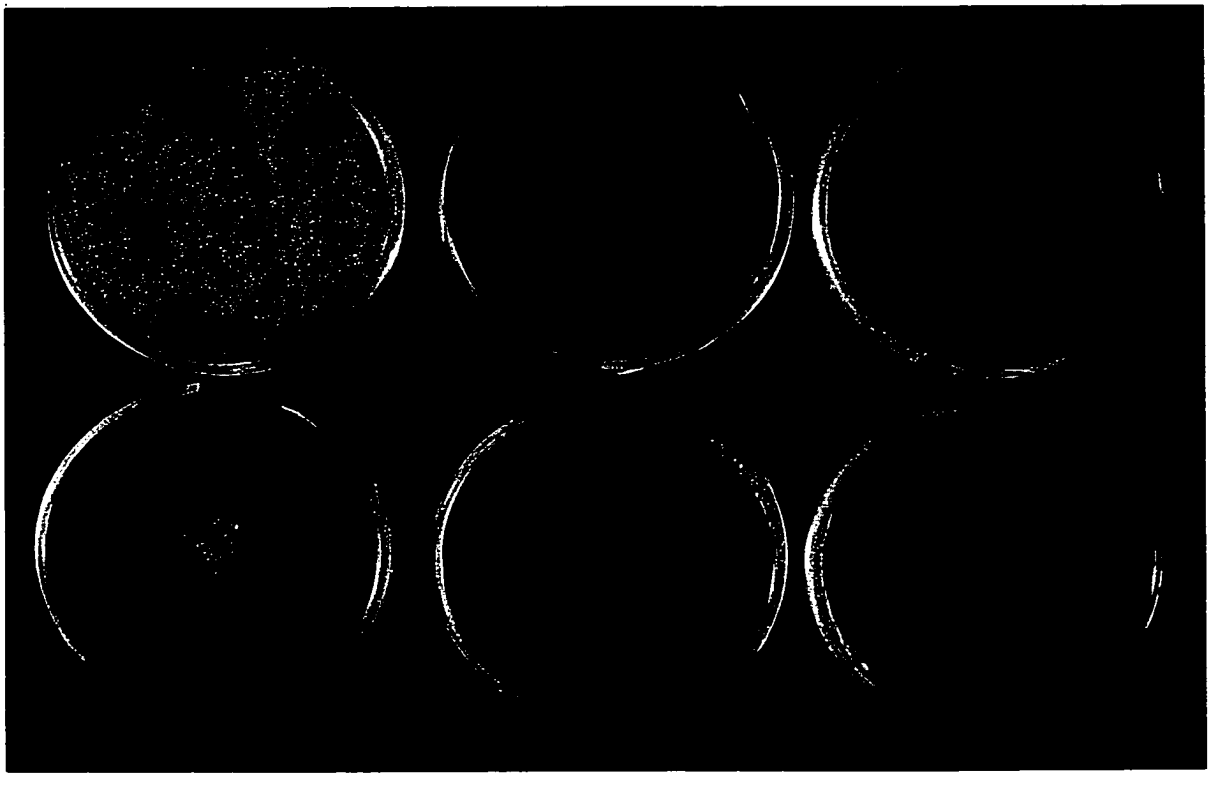
Table 5-3. Specific activities of pooled, concentrated fractions

	Specific Activity (mU/ mg protein)*	
Enzyme	**1 week**	**4 week**
Laccase	7110	1150
Mn P	MnP 1 100	438
	MnP 2 257	
AAO	ND	AAO 1 3.4
		AAO 2 3.2

ND- Not detected

*1 mU = 1 nmol of product/ min/ ml

Figure 5-1. Poly R-478 decolorization plate assay. Top (L-R) *Phanerochaete chrysosporium* UAMH 3642 (positive control), *C. olla* accession PR6, *C. olla* accession Rav., Bottom (L-R) *C. olla* accession PR24, *C. olla* accession DAOM 197577, uninoculated control.



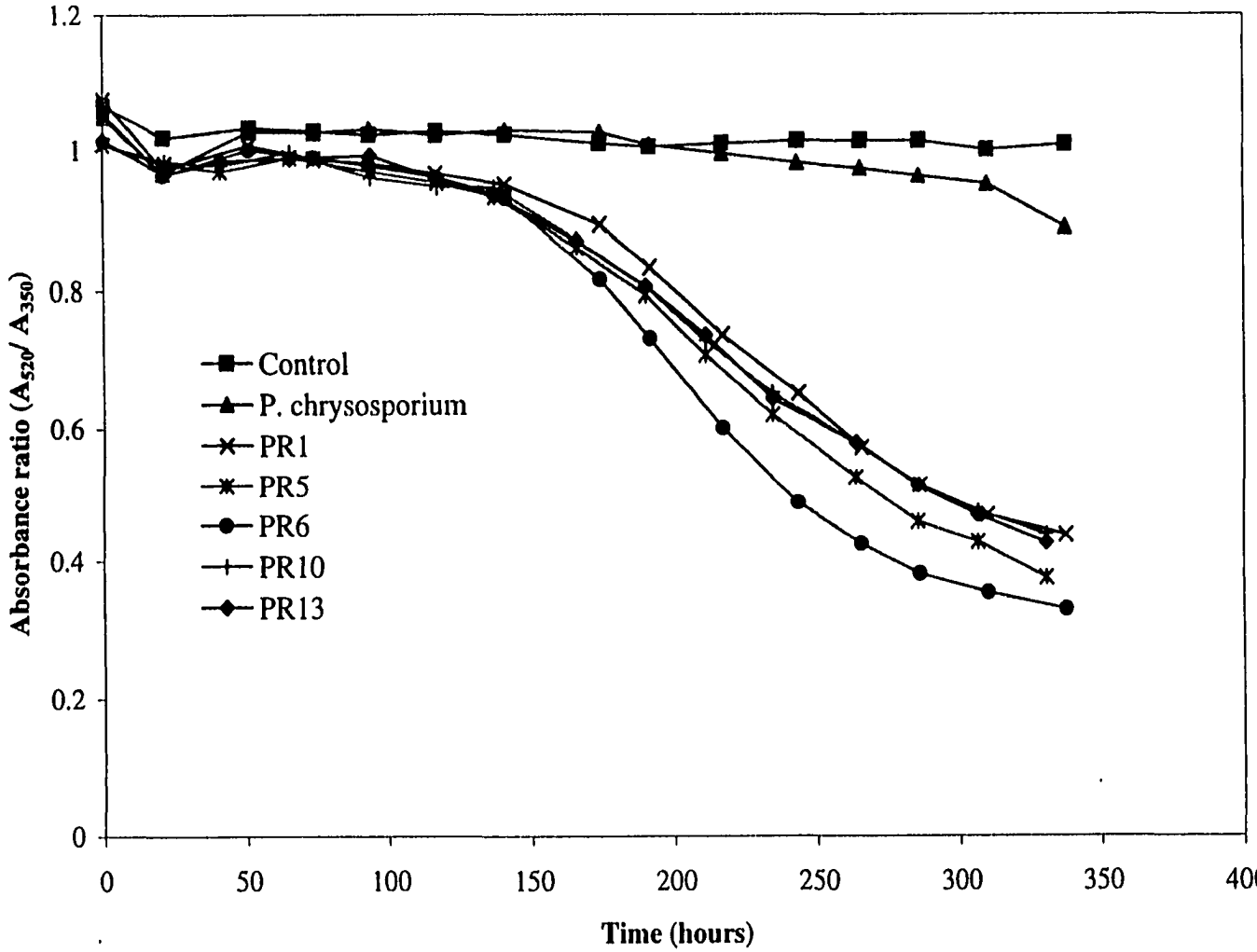


Figure 5-2. Decolorization of Poly R-478 in liquid culture.

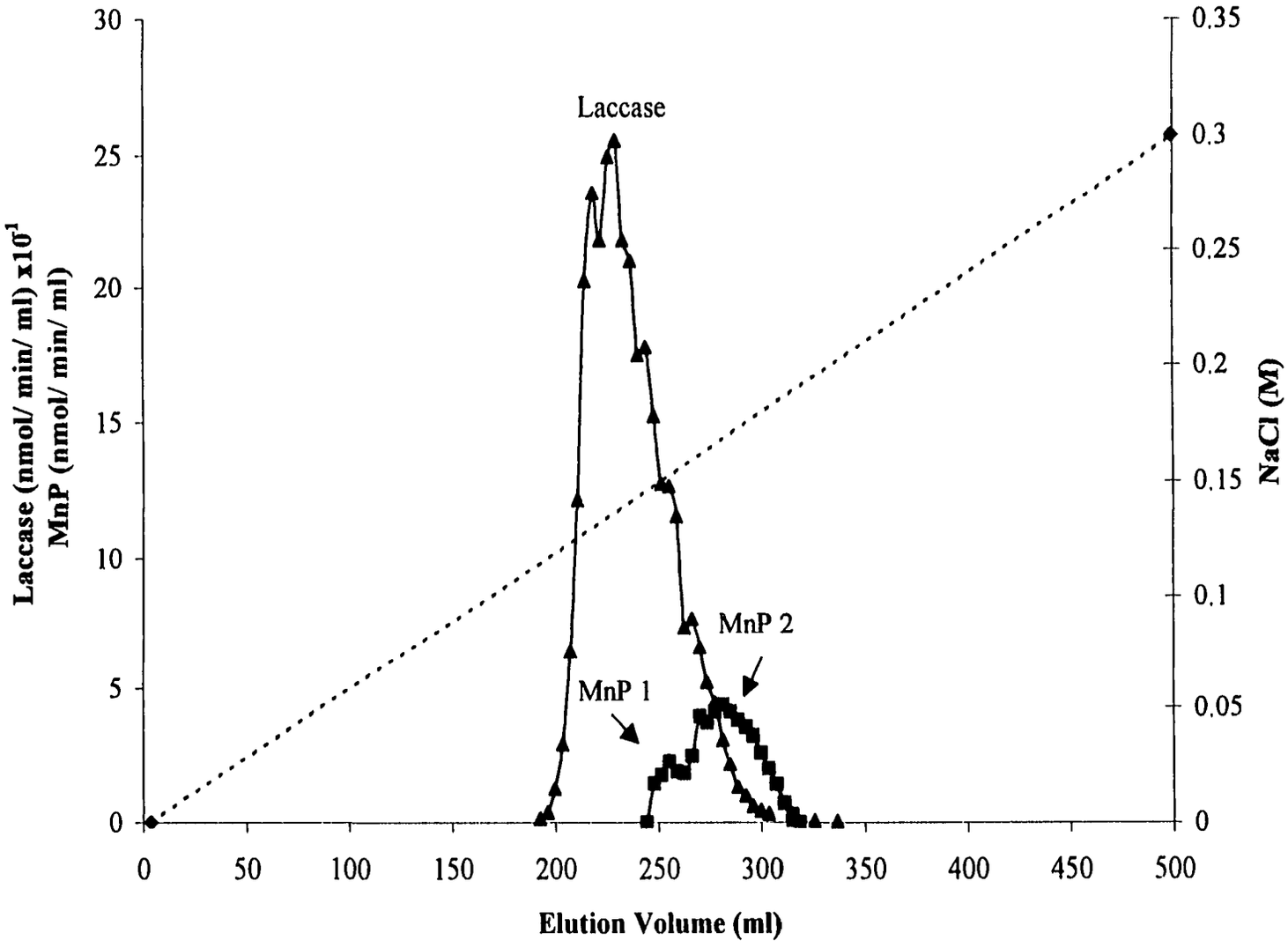


Fig. 5-3. Enzyme elution from an anion-exchange column and activity profile detected from canola root material following 1-wk of solid-state fermentation with *C. olla*.

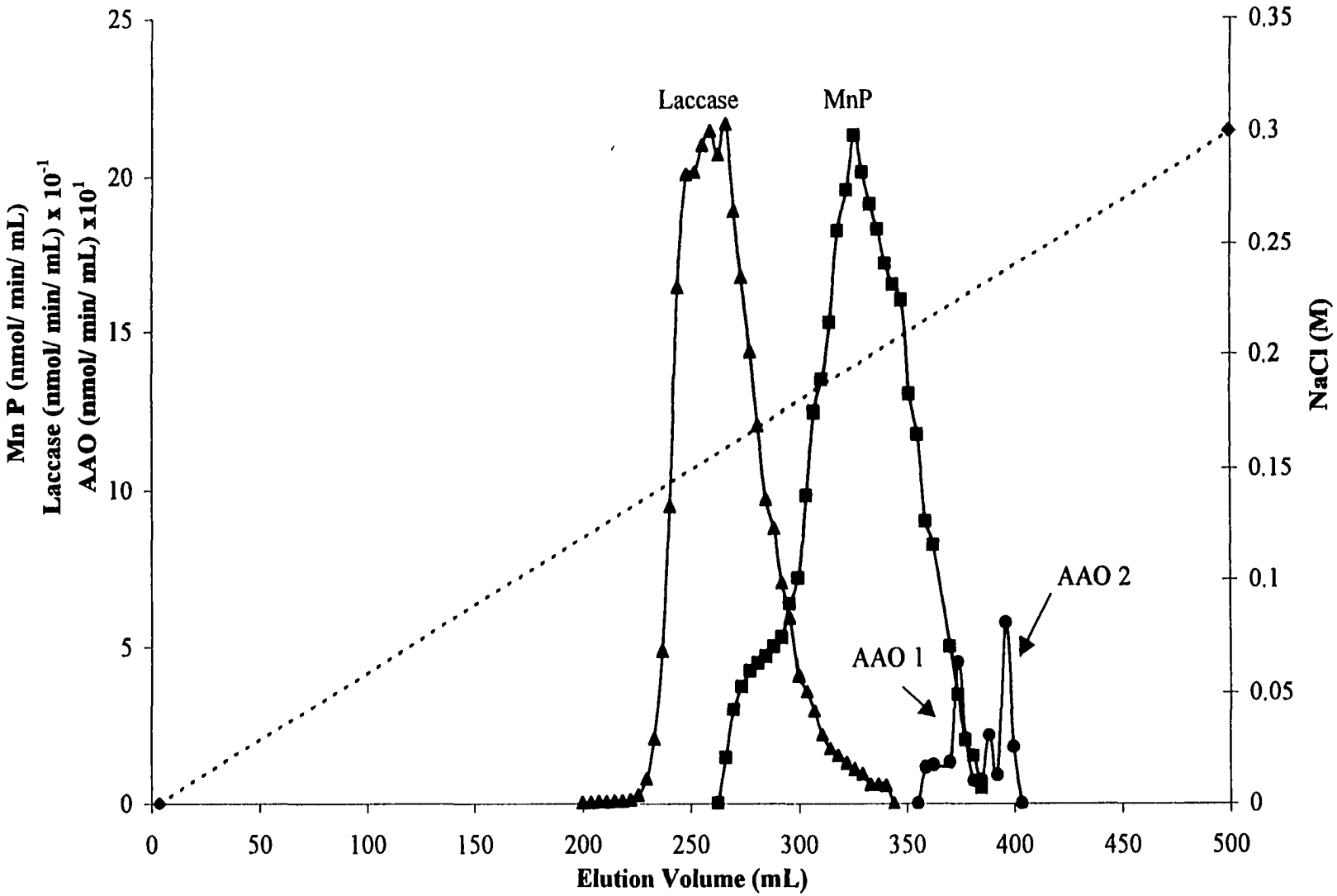


Fig. 5-4. **Enzyme elution profile from an anion-exchange coloumn and activity detected from canola root material following 4-wks of solid-state fermentation with *C. olla*.**

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Chapter 6

Decomposition of canola stubble by solid state fermentation with *Cyathus olla*

6.1 Introduction

In the field, canola stubble infested with *Cyathus olla* appears soft and macerated (Tewari and Briggs, 1995), but the extent of degradation incited by this fungus is not known. Previous studies have reported the ability of *Cyathus* spp. to reduce the lignin content in maple hardwood (Wicklow *et al.*, 1984), and *C. stercoreus* to delignify and improve the digestibility of various plant residues (Akin *et al.*, 1995; Karunanandaa *et al.*, 1992; Chen *et al.*, 1995; Wicklow *et al.*, 1984).

Scanning electron microscopy of field collected *C. olla* infested canola stubble revealed calcium oxalate crystals associated with the colonizing hyphae, and structural deterioration of the substrate (Tewari *et al.*, 1997; Chapter 2). In addition, the lignin degrading enzymes manganese peroxidase, laccase, and aryl-alcohol oxidase have been isolated from canola root material following solid state fermentation with *C. olla* (Shinners-Carnelley *et al.*, submitted; Chapter 5). These results suggested that *C. olla* is capable of lignin degradation, but the extent of this activity must be quantified, and subsequent changes in botanical fractions of canola determined. The basal stem and root of canola are woody, but the proportion of structural cell wall components like lignin, cellulose, and hemicellulose have not been determined. In addition, much morphological and molecular variation has been observed in *C. olla* (Shinners and Tewari, 1998; Chapter 4), and it is not known if the three forms of this fungus commonly found on canola stubble vary with respect to lignin degradation.

The objectives of this study were to determine the structural composition of the basal stem and root of canola, and quantify the changes in cell wall components of this canola substrate following solid-state fermentation with three forms of *C. olla*.

6.2 Materials and Methods

6.2.1 Plant material

Five cultivars of *Brassica napus*, varying in susceptibility to blackleg and straw strength, were selected for this experiment (Table 6-1). These cultivars were selected

since stubble decomposition is targeted for control of blackleg, and it is not known if plant composition is a factor in susceptibility to this disease. No cultivars could be selected in relation to blackspot since all canola cultivars are susceptible. Straw strength rating was also considered as this parameter may influence the quality and quantity of fiber in the plant. Field plots were planted in a randomized block design at the Edmonton Research Station, University of Alberta, Edmonton, Alberta in May, 1998. Following harvest in September, standing stubble was dug from the plots in order to remove the root and stem portions. The stubble was dried at 60°C for 48 h and the stubble pieces were cut and separated into root/ basal stem and main stem pieces. The root and basal stem portions were ground in a Wiley mill.

6.2.2 Compositional analyses

Chopped root/ basal stem material was ground to a fine consistency (approximately 1 mm particle size) in a coffee grinder prior to analyses. The Goering Van Soest (Goering and Van Soest, 1970) method of fiber analysis was performed using the filter bag technique and the Ankom^{200} Fiber Analyzer (ANKOM Company, Fairport, New York). Fractions determined included neutral detergent solubles (NDS), neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, lignin, and hemicellulose, whereby hemicellulose was calculated as the difference between NDF and ADF, and lignin was the fraction remaining following digestion in 72% H_2SO_4 (Klason lignin), minus the ash component remaining following 10 hrs at 500°C. Total nitrogen was determined using the LECO Nitrogen Analysis system (LECO Instruments Limited, Mississauga, Ontario).

6.2.3 *C. olla* accessions and solid-state fermentation

Fifteen *C. olla* accessions, five of each of *C. olla* f. *olla, C. olla* f. *anglicus,* and *C. olla* f. *brodiei,* were used in this study. All accessions were collected from northern and central Alberta, Canada (Table 6-2), and maintained on potato dextrose agar (PDA). Accessions were selected based on the ability to degrade a model lignin substrate (Shinners-Carnelley *et al.*, submitted; Chapter 5).

Fermentations were carried out in 250 mL Erlenmeyer flasks, each containing 3g of ground canola substrate (cv. Cyclone), and moistened with 15 mL of dH_2O. The flasks were closed with a cotton plug, and covered with aluminum foil to prevent moisture loss, then autoclaved for 30 min at 121°C. Sterilization was repeated twice, at 24 h intervals.

The flasks were inoculated with six 2 mm X 2 mm agar plugs cut from 1-wk old PDA plate cultures of the *C. olla* accessions. Three flasks were inoculated with each accession. Three uninoculated flasks served as the control treatment. All flasks were incubated at 25°C in the dark for 45 d. At 10, 20, and 30 d following inoculation, 1 mL of sterile water was added to each flask. After 45 d, the flasks were dried at 100°C for 48 h, and loss in dry matter was determined by calculating the difference in the final dry weight compared to the initial weight prior to fermentation. The contents of each flask were ground in a coffee grinder, and then the compositional and nitrogen analyses were performed, as previously described. Each flask was sub-sampled three times in order to account for uneven colonization of the substrate by the fungus. This entire growth experiment was repeated once.

6.2.4 Statistical analyses

The data obtained were analyzed using the PROC GLM procedure of SAS 6.0, and means were separated using Tukey's test at the 5% level of probability. All values were presented as a percent of the dry matter of each sample.

6.3 Results

6.3.1 Structural composition of canola

Analyses of the five cultivars determined that each was variable with respect to cell wall composition (Table 6-3). The NDF values ranged from 70.1-77.7% of the total dry matter, with the cv. Cyclone having a significantly higher NDF content than the other four cultivars. This cultivar also had the highest percentage of cellulose and hemicellulose, but the cv. Westar had the highest percentage of lignin. Surprisingly, the cv. Q2 had the lowest NDF value (70.1%). This cultivar rates excellent with respect to straw strength, and it was anticipated that it would have a higher proportion of NDF

compared to the other cvs. such as Legacy (74.1 % NDF) and Alto (70.3% NDF) which are rated as good and fair, respectively. Differences in the nitrogen content were statistically significant among the cultivars used in this experiment. However, biologically these differences in N values do not appear to be important considering the small percentage of this fraction (0.9-1.0% N) and the experimental error involved in the methodology.

6.3.2 Solid-state fermentation by *C. olla*

Following solid state fermentation with *C. olla*, the dry matter content of all inoculated material was significantly reduced compared to the uninoculated control (Table 6-4). *Cyathus olla* f. *brodiei* produced the greatest reduction in dry matter followed by *C. olla* f. *olla* and *C. olla* f. *anglicus*. Loss in dry matter for all treatments was adjusted by 5.7% to account for the higher temperature at which the samples were dried following solid state fermentation. The lignin fraction of all the fermented substrate was reduced, and varied significantly between the forms of *C. olla*. Canola incubated with *C. olla* f. *brodiei* had 10.9% lignin compared to 11.5, 12.5, and 15.5% for *C. olla* f. *olla*, *C. olla* f. *anglicus*, and the control, respectively (Table 6-4). These results indicate that *C. olla* f. *brodiei* was the most effective at lignin degradation, as only 60.6% of this fraction remained following fermentation (Table 6-5). *Cyathus olla* was also capable of metabolizing hemicellulose, as this fraction was significantly reduced compared to the control. Cellulose did not appear to be degraded by *C. olla* when calculated as a percentage of the total composition. However, when expressed as a percentage of the original dry mass (Table 6-5) cellulose slightly decreased. The NDS fraction of all inoculated treatments increased significantly compared with the control. This fraction measures cell contents including soluble carbohydrate, starch, organic acids, protein and pectin (Van Soest, 1982), but does not differentiate between plant and fungal tissue. Fungus-mediated breakdown of structural cell wall components increases the soluble carbohydrate content of the NDS fraction, but as the fungi grow, these carbohydrates are incorporated into the fungal biomass.

The N content of the fermented samples did not differ greatly between treatments. Statistically, the canola fermented with *C. olla* f. *olla* had a significantly

higher N content when compared with the other forms of *C. olla* and the control (Table 6-4). However, for the reason discussed previously with respect to N content in the cultivar experiment, this result does not appear to be biologically significant. In addition, the fermented samples also contained fungal biomass, and the N content represented in Table 6-4 does not distinguish between plant and fungal N.

6.4 Discussion

The cultivars examined in this study vary in their tolerance to the blackleg disease and straw strength. The results indicated that variability also exists among these cultivars with respect to structural composition. The composition of the basal stem and root of canola have not been previously reported, but Kohlmann *et al.* (1995) determined that rapeseed stems and siliques were composed of 38 and 35 % cellulose, 10 and 12% hemicellulose, and 18 and 18% lignin on a dry weight basis, respectively. The root and the basal stem of the plant are more resistant to decomposition compared to stems and siliques, and it is assumed that the proportion of lignin and structural carbohydrates would be greater in this more resistant tissue. This assumption is supported by the data obtained for cellulose and hemicellulose content of root and basal stem tissue in the current study. However, these comparisons can only be generalized since they are based on the results of two independent experiments.

Lignin content of the cv. Cyclone was 14.5% in the cultivar experiment, but when this canola tissue was used as the substrate and uninoculated control for the solid state fermentation experiment, the lignin content of cv. Cyclone was determined to be 15.5% of the dry matter. This is not a large discrepancy in data, but may have resulted from the formation of Maillard products. When exposed to heat greater than 60°C, carbohydrates may degrade, resulting in condensation of sugar residues with amino acids, polymerization, and subsequent formation of lignin like compounds, referred to as Maillard products, via the Maillard reaction (Van Soest, 1982). In the fermentation experiment, the substrate was subjected to heat sterilization in order to eliminate other stubble microorganisms that would have interfered with the colonization and decomposition of canola by *C. olla*. During this process, the Maillard reaction may have occurred, resulting in a slightly higher lignin content for the fermented samples.

Fermentation with every form of *C. olla* resulted in a decrease in the lignin fraction of the substrate. This result complements field observations of softened, macerated *C. olla* infested stubble (Tewari and Briggs, 1995; Chapter 2) and the recent report of a lignin degrading enzyme system in this species (Shinners-Carnelley and Tewari, submitted; Chapter 5). Fermented samples had significantly lower lignin contents compared to the control. However, Horwath and Elliott (1996) caution that the Klason method of lignin determination, as used in this experiment, may be a conservative estimate of this fraction.

Fermented samples had a significantly lower hemicellulose fraction compared with the control, indicating that *C. olla* also metabolized this carbohydrate as an energy source during the incubation period. Cellulose concentration in the decayed canola increased, but mass decreased slightly.

As degradation of crop stubble occurs in the field, N is metabolized from the residue and immobilized in the microbial biomass or mineralized and made available for plant growth. Ultimately, this results in a lower N content in the stubble. In this experiment, %N in the fermented samples was not significantly different than the control, with the exception of *C. olla* f. *olla* which had a higher N content following fermentation. As previously mentioned, the methods used in this experiment did not allow for separation of plant and fungal biomass, and subsequently, plant and fungal N.

Bird's nest fungi are white-wood rotters ecologically specialized to degrade lignin. The results obtained here confirmed that *C. olla* is capable of degrading canola *in vitro*, but also demonstrated the variability within this species with respect to stubble degrading capabilities. *Cyathus olla* f. *brodiei* was most effective at reducing the overall dry weight, and lignin and hemicellulose fractions of canola. This is a significant finding since *C. olla* f. *brodiei* has only recently been designated as a new form of *C. olla* (Shinners and Tewari, 1998; Chapter 4). This form is morphologically and molecularly distinct from the other previously described forms, and the results reported here demonstrated that this fungus is also biochemically unique.

These results support the proposed use of *C. olla* as a stubble inoculant intended to accelerate canola stubble decomposition, but in order to confirm these findings, field testing must be conducted to assess decomposing activity under natural conditions. The

activity of *C. olla* in the stubble microenvironment and the ecological interactions with other stubble colonists, must be understood before this fungus can be developed as a stubble inoculant.

6.5 Tables

Table 6-1. *Brassica napus* cultivars used in this study

Cultivar	Tolerance to blackleg[a]	Straw strength
Q2	1	Excellent
Cyclone	2	Very good
Legacy	3	Good
Alto	5	Fair
Westar	5	Fair

[a]Blackleg tolerance, 1=tolerant, 2=moderately tolerant, 3=moderately susceptible, 4=susceptible, 5=highly susceptible.

Table 6-2. *Cyathus olla* accessions used in this study

Form	Accession ID	Collection location
C. olla f. *olla*	PR 5	Canada, Alberta, NW 9-78-21-5 [b]
C. olla f. *olla*	PR 6	Canada, Alberta, SW 35-79-22-5 [b]
C. olla f. *olla*	PR 10	Canada, Alberta, SE 5-78-21-5 [b]
C. olla f. *olla*	PR 15	Canada, Alberta, SW 2-79-22-5 [b]
C. olla f. *olla*	Beau	Canada, Alberta, Beaumont
C. olla f. *anglicus*	Ravine	Canada, Alberta, Edmonton
C. olla f. *anglicus*	PR 1	Canada, Alberta, SE 12-79-21-5 [b]
C. olla f. *anglicus*	PR 18	Canada, Alberta, NW 16-77-21-5 [b]
C. olla f. *anglicus*	PR 22	Canada, Alberta, SE 6-78-20-5 [b]
C. olla f. *anglicus*	PR 25	Canada, Alberta, NE 33-77-20-5 [b]
C. olla f. *brodiei*	PR 3	Canada, Alberta, SW 24-78-20-5 [b]
C. olla f. *brodiei*	PR 7	Canada, Alberta, SW 6-80-20-5 [b]
C. olla f. *brodiei*	PR 19	Canada, Alberta, NW 10-77-21-5 [b]
C. olla f. *brodiei*	PR 23	Canada, Alberta, Falher
C. olla f. *brodiei*	PR 27	Canada, Alberta, SE 26-78-21-5 [b]

[b] Legal descriptions within the Municipal district of Smoky River, Alberta, Canada

Table 6-3. Composition of root and basal stem tissue of *Brassica napus* cultivars

	Q2	Cyclone	Legacy	Alto	Westar	Standard Error
% of Dry Matter						
NDF [c]	70.1 b	77.7 a	74.1 ab	70.3 b	72.0 b	0.51
NDS[d]	28.4 a	21.2 b	24.8 ab	28.4 a	27.1 a	0.43
ADF [e]	52.8 c	59.1 a	56.4 ab	53.6 bc	55.6 abc	0.14
Cellulose	39.4 b	44.5 a	43.3 a	39.4 b	40.1 b	0.35
Lignin	13.4 c	14.5 b	13.1 c	14.2 b	15.5 a	0.14
Hemicellulose	17.3 bc	18.6 a	17.7 ab	16.7 c	16.5 c	0.18
Ash	1.3 a	0.91 bc	0.87 bc	1.1 ab	0.63 c	0.05
% N	1.0 a	0.90 b	0.90 b	1.0 a	0.90 b	0.02

[c] NDF, Neutral detergent fiber

[d] NDS, Neutral detergent solubles

[e] ADF, Acid detergent fiber.

Means with the same letter within rows are not significantly different (p=0.05).

Table 6-4. Chemical composition of canola following 45 d solid state fermentation with three forms of *Cyathus olla*

	Control	*C. olla* f. *anglicus*	*C. olla* f. *olla*	*C. olla* f. *brodiei*	Standard Error
% of Dry Matter					
Loss in dry matter	0.0 a	11.5 b	11.9 b	14.3 c	0.20
Lignin	15.5 a	12.5 b	11.5 c	10.9 d	0.15
Hemicellulose	19.0 a	17.4 b	16.8 c	16.6 c	0.16
Cellulose	45.6 a	47.5 b	47.6 b	47.9 b	0.24
NDS [f]	18.7 a	21.3 b	22.5 c	23.4 d	0.29
Ash	0.85 a	0.94 a	1.3 b	0.8 a	0.07
% N	0.94 b	0.95 b	1.1 a	0.91 b	0.02

[f]NDS, Neutral detergent solubles

Means with the same letter within rows are not significantly different (p=0.05).

Table 6-5. Chemical composition of canola expressed as a percentage of original dry mass following 45 d solid state fermentation with three forms of *Cyathus olla*

	Control	*C. olla* f. *anglicus*	*C. olla* f. *olla*	*C. olla* f. *brodiei*
% of Original Dry Mass				
Loss in dry matter	0.0 a	11.5	11.9	14.3
Lignin	100	71.8	65.9	60.6
Hemicellulose	100	81.6	78.6	75.3
Cellulose	100	92.8	92.8	90.6
NDS [g]	100	101.5	106.9	107.9
Ash	100	98.5	135.9	81.1
% N	100	90.0	104.0	83.5

[g]NDS, Neutral detergent solubles

6.6 References

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Chapter 7

7.1 General Discussion

Cellulose and lignin, respectively, are the most abundant organic compounds on earth (Salisbury and Ross, 1991). Lignin and cellulose are complexed together in plant cell walls to form lignocellulose which provides strength and protection from microbial attack. Organic matter decomposition by bacteria and fungi is commonly limited by the abundance of this recalcitrant material, as only a small proportion of microbes are capable of degrading lignin. Many saprophytic fungi can enzymatically degrade cellulose and hemicellulose, but these enzymes cannot access the target substrate unless lignin is depolymerized and removed (Blanchette, 1991). White-rot fungi are ecologically specialized to perform this function.

Crop residues high in lignocellulose resist decomposition, and can remain in the field for an extended period of time. During this time, stubble may serve as an overwintering site and food source for plant pathogens and become an important component of the disease cycle. Elimination of stubble by tillage or burning provides control for stubble-borne diseases, but these practices are not considered sustainable, and alternative, innovative methods to reduce crop stubble should be developed.

The ultimate objective of this thesis was to assess the potential of *Cyathus olla*, a white wood-rotting fungus, at being developed into a microbial inoculant to accelerate canola stubble decomposition. The incidence of stubble-borne diseases of this crop may be reduced by eliminating the overwintering site and food source for pathogens. It was not the intention to develop this inoculant, but rather to build a body of knowledge on some biological and biochemical attributes of *C. olla*. Brodie (1975) studied the biology of the bird's nest fungi in detail, but there is limited information on this group of fungi in applied research. In order to determine if *C. olla* would be feasible as a microbial inoculant for the proposed use, some basic questions needed to be addressed. The research conducted and results presented in this thesis answer some of these questions, and support future research on *C. olla* as a microbial stubble inoculant.

7.1.1 Oxalic acid and calcium oxalate crystal production

The initial report of infestation of canola stubble by *C. olla* indicated the presence of crystals, typical of calcium oxalate, encrusting the colonizing hyphae (Tewari and Briggs, 1995). Accumulations of calcium oxalate crystals on the hyphae of basidiomycetes are commonly reported to be associated with decomposing plant tissue (Connolly and Jellison, 1995; Graustein *et al.*, 1997), but none had been reported for *Cyathus* spp. For this reason, scanning electron microscopy studies of *C. olla* infested canola stubble and *C. striatus* infested wood chips were conducted to detect and identify crystals associated with the hyphae of these fungi when colonizing a natural substrate, and when grown on artificial media.

Hyphae of *C. striatus* and *C. olla* in culture and on infested plant debris were heavily encrusted with crystals. Scanning electron microscopy revealed that raphide- and styloid- shaped crystals were associated with the hyphae of *C. olla* in canola stubble and in culture. Bipyramidal crystals were also present in culture. Distinct raphide druses developed on *C. striatus* hyphae colonizing wood chips, but in culture most crystals were bipyramidal or other shapes. Energy-dispersive X-ray microanalyses, FT/ IR spectroscopy, and ^{13}C NMR spectroscopy determined that these crystal were calcium oxalate. The secretion of oxalic acid and formation of calcium oxalate crystals by these fungi has implications for decomposition of organic matter, biomineralization, and nutrient cycling.

Oxalic acid is secreted by many ascomycetes, deuteromycetes, and basidiomycetes, and is typically associated with pathogenesis and lignocellulose degradation (Dutton and Evans, 1996). When secreted by fungal pathogens during pathogenesis, oxalic acid serves to acidify host tissues and optimizes the pH for polygalacturonase activity (Bateman and Beer, 1965). As well, oxalic acid sequesters calcium from cell walls of the host and chelates with it to form calcium oxalate crystals (Bateman and Beer, 1965). In plant tissues, calcium is complexed with pectic substances (calcium pectate) in the middle lamella of cell walls and inhibits enzymatic activity (Bateman and Lumsden, 1965). Once calcium is removed, plant tissues are susceptible to enzymatic degradation. Oxalic acid has been shown to be synergistic with polygalacturonase (Bateman and Beer, 1965).

When secreted by wood-rotting fungi, oxalic acid may function in the same role as described for pathogenesis, but has also been implicated in the regulation of the lignin degrading enzyme system in the white-rot fungi (Shimada *et al.*, 1994). Depolymerization of lignin occurs predominantly due to the activity of two peroxidase enzymes, lignin peroxidase, and manganese peroxidase; laccase; and H_2O_2- generating oxidases (Kirk and Farrell, 1987). Recently, oxalic acid at physiological concentrations has been shown to stimulate the activity of manganese peroxidase from the white-rot fungus *Phanerochaete chrysosporium* (Kuan and Tien, 1993). Urzua *et al.* (1998) have also shown that the oxidation of oxalic acid by manganese peroxidase results in the formation of H_2O_2, a co-substrate for both lignin peroxidase and manganese peroxidase. Peroxide-generating enzymes cannot always be isolated from ligninolytic fungi, and oxalic acid may play a significant role in enzyme function in the absence of these oxidase enzymes.

In the environment, oxalic acid and oxalate play a critical role in the availability of nutrients. Cromack *et al.* (1977) have suggested that the formation of calcium oxalate crystals by fungi serves an important role in calcium recycling by maintaining calcium in the root zone, and preventing leaching of this nutrient. Calcium oxalate is sparingly soluble, but is degraded by bacteria to recycle the cation, and then forms calcium carbonates or bicarbonates.

Secretion of oxalic acid weathers mineral soils since the oxalate anion is an effective weathering agent (Griffiths *et al.*, 1994; Graustein *et al.*, 1977). Increased soil weathering enhances the availability of nutrients for plant uptake. Griffiths *et al.* (1994) have determined that concentrations of dissolved organic C, PO_4, SO_4, H, Al, Fe, Cu, Mn, and Zn were significantly higher in forest soils that contained fungal mats of *Hysterangium setchellii* and *Gautieria monticola* compared to non-mat soils. Oxalate can also affect the relative solubility of Fe and Al by forming stable complexes with these trace metals, and can either inhibit precipitation or enhance the dissolution of Fe and Al phosphates. In doing so, the chelation of Al and Fe releases P and improves the availability for plant uptake (Griffiths *et al.*, 1994; Graustein *et al.*, 1977). Fox *et al.* (1992) have shown that oxalate loading of soils directly influenced the availability of P in subsoils. Oxalate in soil replaces P bound to Al-oxide surfaces, and also enhances

the dissolution of Al-oxide surfaces. The Al released as a result of this exchange complexes with oxalate, reducing free metal activity, and the potential of metal toxicity to plants (Hue *et al.*, 1986).

Examination of the 11 additional Nidulariaceae species from culture identified eight of these species as calcium oxalate crystal forming fungi, and contributed to the number of fungal species known to produce calcium oxalate crystals. More crystals were produced on the calcium rich V8 juice agar than on PDA, and no association was found between the crystal types and geographic and habitat distribution of species. The taxonomy of the Nidulariaceae is based on basidiocarp morphology, but fruiting bodies are not always available. Because artificial induction of fruiting is difficult, and is only successful for a few species, criteria for hyphal characterization and species identification would be helpful. It is hoped that crystal morphology could be used as one parameter to aid in hyphal identification of the bird's nest fungi. In many plants, crystal shape and location are species and tissue specific (Horner and Wagner, 1995), but to date, this has not been shown for the Nidulariaceae.

7.1.2 Morphological and molecular characterization

In order to conduct the experiments documented in this thesis, a collection of bird's nest fungi was developed. All basidiocarps collected from agricultural fields in northern and central Alberta were identified as *C. olla*. At the onset of this project, *C. olla* and *C. striatus* were both being considered as possible candidates for a microbial inoculant, but the prevalence of *C. olla* in field collections turned the focus to this one species. *Cyathus olla* appears to be adapted to agricultural environments since it is naturally found growing and fruiting on canola stubble (Tewari and Briggs, 1995), and can be collected from the same field for many years. In a field survey conducted by Baird *et al.* (1993), *C. olla* was found to be one of the most common fungal species of fleshy fungi in agricultural fields in Georgia, USA. Although *C. striatus* is common in the same climatic zones as *C. olla, C. striatus* has not been found in agricultural environments. For this reason, *C. striatus* was not pursued in further research for the eventual development as a stubble inoculant.

Initial examination of *C. olla* basidiocarps showed much variation among the various collections, and identified morphological characters that conflicted with the present taxonomic description of the species. Based on these observations, all field-collected accessions were characterized on the basis of basidiocarp morphology. As well, PCR-based RAPD analysis distinguished among the accessions, and UPGMA analysis generated a dendrogram consisting of three distinctly branched groups. The accessions within this collection represented three forms of *C. olla* including, *C. olla* f. *olla, C. olla* f. *anglicus,* and a previously undescribed plicate form. Morphological and RAPD data collected throughout this experiment supported the distinction of a new plicate form that was described and named *C. olla* f. *brodiei.*

The RAPD-PCR technique is a new tool commonly used for producing characters for fungal systematics, and provides useful resolution at the intraspecific level (Kohn, 1992). A weakness with this technique is the assumption that co-migrating fragments are homologous to each other (Weising *et al.,* 1995). It is not feasible to test for homology in this type of analysis where many RAPD markers are typically produced. For this reason, statements of variability within a species, based solely on RAPD data, may be controversial. However, these data become significant when used in conjunction with other taxonomic criteria, such as the morphological characters used in this study.

Dr. H.J. Brodie described many bird's nest fungi throughout his career, and noted much intraspecific variability (Brodie, 1975). He refrained from justifying most of this variability taxonomically, but in a taxonomic key published in 1977 in which he addressed the variability of *C. olla,* Brodie stated that the forms of this species were not plicate (Brodie, 1977). Since that time, no additional forms or species have been described. Because of this, the plicate accessions warranted further examination, and the eventual designation as a new form of *C. olla.*

7.1.3 Plant cell wall degrading enzymes

Field observations of *C. olla* colonizing canola stubble (Tewari and Briggs, 1995) and the detection of calcium oxalate crystals associated with colonizing hyphae (Tewari *et al.,* 1997: Chapter 2 & 3) suggest that this fungus is actively involved in the

decomposition of canola stubble. *Cyathus olla* was known to be a white wood-rotting fungus, but the enzyme system had not been studied in detail. For this reason, the plant cell wall degrading enzymes produced by *C. olla* during solid state fermentation of canola root material for 1- and 4- wk incubation periods were studied. Anion exchange chromatography and other assays enabled the fractionation and identification of ligninolytic enzymes. Laccase and manganese peroxidase were detected from both 1-wk and 4-wk incubations, and aryl-alcohol oxidase was also detected from the 4-wk incubation. Crude buffer extracts were assayed for cellulases and polygalacturonase, but only the latter was detected. The detection of polygalacturonase activity from colonized canola stubble was a significant finding since the literature contains few reports of pectinolytic enzymes from wood-rot fungi (Sethuraman *et al.*, 1998; Green *et al.*, 1995). Because pectin is found in small amounts in wood, pectinolytic enzyme activity by wood-rotting fungi has generally been overlooked.

The detection and identification of plant cell wall degrading enzymes from *C. olla* demonstrated that this fungus has the enzymatic potential to degrade lignin, and supports the use of *C. olla* as an inoculant to accelerate stubble decomposition. This is the first report of laccase, manganese peroxidase, and polygalacturonase production and activity from this fungus. Little is known regarding the enzyme system of *C. olla*, and in order to pursue this fungus for further development as a stubble inoculant, the optimum conditions for enzyme production need to be determined.

7.1.4 Canola degradation by *C. olla*

Chapter 6 addressed a fundamental question of this research. Does *C. olla* degrade canola stubble? The results presented in chapters 2 and 5 suggest that degradation of canola stubble is possible, but it was important to detect and quantify the changes in botanical fractions of canola following incubation with the fungus. In order to do this, the structural composition of canola was also determined, and served as the uninoculated control for this experiment. All canola cultivars examined were variable with respect to cell wall composition and overall fiber content. Following a 45-day incubation of canola root with the three forms of *C. olla*, the dry weight, and lignin and hemicellulose fractions of the ground, decayed canola tissue were significantly reduced

compared to the uninoculated control. *Cyathus olla* f. *brodiei* was the most effective at delignifying the substrate. This is a significant finding which indicates that this form may be better suited for further development, however this must be confirmed with more research.

The ability of *C. olla* to degrade the lignin fraction of canola root during solid state fermentation verifies the previous results presented that suggested that *C. olla* had the potential to degrade this structural polymer. The production of calcium oxalate crystals (Chapter 2) and the detection of a ligninolytic system in *C. olla* (Chapter 5) provided strong support for this function, but was circumstantial prior to the compositional analysis. Decomposition of crop residues is a complex process, and it was, by no means represented by the solid state fermentation conducted in this experiment. This experiment was designed strictly to determine if *C. olla* degraded canola stubble. The positive results of this study strongly support and encourage further study to examine the role of *C. olla* in the decomposition of canola stubble under field conditions, and in the presence of other stubble inhabitants.

Cellulase activity was not detected in these experiments. From the compositional analysis, the cellulose concentration of canola increased following incubation with this fungus, but the overall mass slightly decreased. The caging of cellulose microfibrils by lignin polymers provides strength and protection from degradation by microorganisms, except for the white-rot fungi which are ecologically specialized to degrade lignin. As lignin is degraded, cellulose becomes exposed. The ability of white-rot fungi to improve the availability of cellulose from lignocellulosic material has led to research to evaluate the potential of these fungi at improving the nutritional value of crop residues as livestock feeds (Chen *et al.*, 1995; Karunanandaa *et al.*, 1995).

The results presented in this thesis indicated that *C. olla* has the biochemical attributes necessary to degrade canola stubble. However, the *in vitro* nature of this study imposes a limitation on extrapolating these data to the field. In order to do this, *in situ* studies must be conducted. Information is needed pertaining to the activity of *C. olla* in the stubble environment, and the interactions with pathogens, other microbes, and crop plants. It is anticipated that increased population levels of *C. olla* will lead to

colonization and accelerated decomposition of residues. Elimination of the food source and habitat of pathogens would reduce inoculum levels, and hence the incidence of stubble-borne diseases. In addition to disease management, accelerated stubble decomposition may also contribute to improved nutrient cycling through mineralization of essential plant nutrients tied up in the surface residues.

7.1.5 Biological control in the stubble microenvironment

Biological control strategies for plant diseases generally involve antagonism of the pathogen by antibiosis, competition, parasitism, or predation, and are targeted at protecting the plant from attack (Baker and Cook, 1974). A less common approach is to target the residue habitat (Bockus and Shroyer, 1998). The unique strategy introduced in this thesis is an example of this type of innovative approach. The literature contains few reports of biological control strategies for stubble-borne diseases, but some have been successful at the experimental level. Pfender *et al.* (1993) were successful at reducing the inoculum of the stubble-borne pathogen *Pyrenophora tritici-repentis,* causal agent of tan spot of wheat, by applying *Limonomyces roseipellis* to wheat straw in conservation tillage plots. The exact mechanism of antagonism has not been confirmed, but *L. roseipellis* is chitinolytic and can degrade the hyphae of *P. tritici-repentis.*

Fernandez (1992a) reduced the incidence of *Fusarium graminearum, Macrophomina phaseolina,* and *Glomerella glycines* infesting soybean residue by applying a conidial suspension of *Trichoderma harzianum.* In another study, *T. harzianum* reduced the incidence of *Cochliobolus sativus* on wheat residue, and *F. graminearum* and other *Fusarium* spp. on wheat and black oat residues (Fernandez, 1992b). In these experiments, *T. harzianum* had no effect on residue decomposition, but may have affected the ability of pathogens to colonize the residue during their saprophytic stage.

7.1.6 Development of *C. olla* as a microbial inoculant

The development of a biological control agent involves many challenges including environmental competence of the agent, determination of formulation,

production techniques, sufficient and consistent efficacy, and registration (Boland, 1990; Cook, 1996). In addition, Pfender *et al.* (1993) have suggested that the development of biological control agents for the residue microenvironment are faced with additional challenges including adequate coverage of the residue with small amounts of inoculum, retention of biological control agents in the residue, and the ability of agents to withstand fluctuations in moisture. Residue inhabiting microbes may be best suited to development since they are adapted to the environmental conditions in this niche. The development of *C. olla* as a microbial inoculant may eventually face all of these challenges, but it is too early to speculate on the success of this disease control strategy. However, there are many indications that this organism will be successful at accelerating canola stubble decomposition.

Cyathus olla appears to be competent in the stubble environment since it has been collected for many years from the same field, and is able to withstand common agricultural practices such as tillage and herbicide applications. If successful, formulation of this microbial inoculant will require much research in order to determine method, quantity, timing, and type of application. Volume of the inoculant to be used should be minimal, and steps should be taken to maximize the shelf life of the product. One way to produce and deliver this type of inoculant could be by inoculating, chopping, and spreading *C. olla* colonized straw. This could be advantageous since straw is a readily available and relatively inexpensive material that is easily colonized by *C. olla,* without the addition of other vitamins or nutrients. Colonized straw could be chopped into fine pieces, and spread on fields using an implement adapted to a seeder or combine. Incorporating the application of a biological control agent into other field practices is advantageous since it does not require any extra time, fuel costs, labor, or field passes.

An appropriate time for applying this inoculant may be in the spring following a canola crop. Under conservation tillage management, canola stubble would be plentiful and provide an abundant source of nutrients for *C. olla.* In conventionally tilled fields, most canola stubble would be buried and not obvious, but the woody root and basal stem of the plant is resistant to decomposition and often resurfaces, or is located just beneath the soil surface. The composition of canola stubble at this stage is not known,

but it is assumed that other saprophytes would have degraded the available sugars, proteins, pectins, and other easily degraded components, and the remaining basal stem and root of canola would be predominantly lignocellulose. The moist spring soil conditions may encourage colonization and establishment of *C. olla* on and in the canola stubble, and the spring and summer temperatures in the months following application would favor growth and development of the fungus.

The efficacy of this type of microbial inoculant is not known, and cannot be speculated upon until field tests are conducted. However, a major challenge for this type of product is to achieve sufficient and consistent efficacy. Biological control agents, by their inherent nature, are variable, and can potentially perform differently over time and under different environmental conditions. The goal of this microbial inoculant is to accelerate canola stubble decomposition, but it is not expected to eliminate stubble shortly after application. If the retention time of stubble could be reduced by one year, producers could safely shorten the rotation between canola crops. This may not seem significant, but it would be beneficial for those producers who would realize increased economic returns for the production of canola, instead of other lower valued crops like wheat or barley. The nutrients within the decomposing residues would be made available for plant uptake, and the growth of *C. olla* in the stubble environment may improve nutrient availability by secretion of oxalic acid. Soil structure may also benefit from increased populations of *C. olla,* as hyphal cords forage through the soil and entangle soil particles into aggregates. *Cyathus olla* may be effective at the proposed use, but the ultimate factor influencing production and development of this technology will be profitability.

The innovative strategy for disease management discussed in this thesis has been developed from observations of natural interactions occurring in the agroecosystem. Research directed at these interactions may lead to an improved understanding of the ecological processes occurring naturally, and provide an ecologically based approach to pest management. The proposed strategy of accelerated stubble decomposition has been studied with respect to canola for the purpose of this thesis. However, the underlying concept of this control strategy is not crop or pathogen

specific, and has the potential to become an important component of an integrated approach to crop and disease management.

7.2 Conclusions

1. *Cyathus olla* and *C. striatus* produce oxalic acid, sequester calcium from their substrata, and form calcium oxalate crystals.

2. Much morphological and molecular variability exists within the species *C. olla*. A new form of this species was described and named as *C. olla* f. *brodiei*.

3. Three forms of *Cyathus olla* (f. *olla*, f. *anglicus*, and f. *brodiei*) are frequently found colonizing the basal stem and root of canola in northern and central Alberta.

4. *Cyathus olla* produces manganese peroxidase, laccase, aryl-alcohol oxidase, and polygalacturonase when grown on ground canola root.

5. *Cyathus olla* reduced the dry weight and hemicellulose and lignin content of ground canola root. *Cyathus olla* f. *brodiei* was the most effective at reducing these fractions.

7.3 Future Considerations

Future considerations for this research should include continued study of the biochemical attributes of *C. olla*, and the collection of ecological information on how *C. olla* establishes and interacts in the stubble environment. This knowledge is imperative for carrying out field applications of this fungus, monitoring the establishment and growth of introduced populations, and determining the most effective time for application of a microbial inoculant.

1. Develop identification and quantification methods for isolation of *C. olla* from stubble.

In order to conduct field studies on stubble ecology in relation to *Cyathus*, a method must be developed to easily identify and quantify fungal biomass. This fungus does not fruit easily, and isolations from stubble pieces are often limited to hyphae or hyphal cords. For this reason, methods must be developed to identify *C. olla* based on hyphal characteristics. This information could be used to develop a hyphal identification system. The development of a molecular probe would also be beneficial for identification.

2. Study the microbial succession on canola stubble and the role of *C. olla* in this process.

Ecological studies must determine the activity of *C. olla* in the stubble environment, and characterize the interactions taking place between this fungus and stubble-borne pathogens, parasites, and other stubble colonizing saprophytes. Research must also determine the resource capture and establishment strategies of *C. olla*, when *C. olla* colonizes canola stubble in the field, and if *C. olla* can effectively colonize pathogen-infested stubble.

3. Define the histology and composition of canola stubble.

The histology of canola stubble has not been determined. In order to have a complete understanding of the colonization of canola stubble by *C. olla*, the substrate should be well defined.

4. Conduct field testing of *Cyathus olla* mediated canola stubble decomposition.

Field studies must be conducted to quantify decomposition of canola stubble in the field under natural conditions and populations of microorganisms, and compare this with decomposition when increased populations of *C. olla* are applied.

5. Study the genetic basis of the ligninolytic enzyme system in *C. olla*.

Cyathus olla has been shown to produce manganese peroxidase, laccase, and aryl-alcohol oxidase. Molecular biological techniques can be used to conduct an in depth study of the enzyme system, and determine the genes that code for these proteins. The ability of *C. olla* to produce this complement of ligninolytic enzymes may also be beneficial to other applications. The degradative enzymes produced by white-rot fungi are secreted extracellularly, are not lignin specific, and can successfully oxidize aromatic rings. For this reason, many white rot species are being evaluated for their potential at degrading recalcitrant compounds (Evans *et al.*, 1998) like triphenylmethane dyes (Vasdev *et al.*, 1995), energetic compounds (Bruce, 1998; Bayman *et al.*, 1995), pesticides (Maloney, 1998; Tuomela *et al.*, 1999), and other polyaromatic hydrocarbons (Kotterman *et al.*, 1998).

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