Biorational potential, pectinase activity, and correlations to virulence among isolates of *Chondrostereum purpureum*.

by

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ABSTRACT

The basidiomycete *Chondrostereum purpureum* is a white-rot fungus which is pathogenic to many deciduous species including poplar, maple, and alder. A phytotoxin activity assay was developed to assist in developing *C. purpureum* as a weed biocontrol agent. Analysis of small mass toxins produced by *C. purpureum* was carried out utilizing several techniques. An important consideration in the development of a mycoherbicide is the elucidation of phytotoxic factors involved in the pathogenic process. Many plant pathogenic fungi are known to secrete lipid soluble toxins which break down cell membrane stability causing host cell necrosis and helping the pathogen to establishing disease. Though no dominant phytotoxic chemical (or chemical group) was readily detected within culture filtrates of the fungus, small mass toxins were confirmed to be produced. Through the use of an excised leaf disk assay and an electrolyte leakage assay, foliage active phytotoxins were detected in ethyl acetate extracts of *C. purpureum*-conditioned media. There was an apparent decrease in overall activity when phenolic fractions were purified. The amount of small mass toxins detected in various isolates of *C. purpureum* did not appear to vary.

Chondrostereum purpureum also produced a pectin degrading enzyme known as endopolygalacturonase. Endopolygalacturonase has been shown to be a key enzyme in the degradation of plant cell walls, and is the causal agent of silver leaf disease. Pectinases produced by the fungus, like endopolygalacturonase, were shown to play a major role in the phytotoxicity of *C. purpureum*. Using leaf disk, electrolyte leakage, and viscosity assays, pectinase production by several isolates of the fungus was measured, and the pectinase levels were found to be correlated to virulence of these isolates towards red alder saplings. There was a strong correlation between pectinase activity of various isolates of *C. purpureum* and the initial slopes of the mortality rates by these same isolates (P-value = 0.0306 by ANOVA). Of twelve selected isolates of *C. purpureum*, the ones that produced higher levels of pectinases in liquid culture were also more virulent in greenhouse pathogencity tests.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
approx.	approximately
bp	base pair
cm	centimetre
cm^2	cm squared
coeff.	coefficient
DNSA	dintrosalicylic acid
exoPG	exopolygalacturonse
endoPG	endopolygalacturonase
e.g.	example
EtOAc	ethyl acetate
et.al.	and others
EL	electrolyte leakage assay
g	gram
g	acceleration of gravity (m/s^2)
GH	greenhouse assay
HPLC	high performance liquid chromatography
HCl	hydrochloric acid
i.e.	ideally
IVM	integrated vegetation management
kDa	kilodalton
LD	leaf disk assay
m	metre
m^3	cubic metre
mM	millimolar
M	molar
MeOH	methanol
mL	millilitre
mg	milligram
mS	millisiemen
N/A	not applicable
nM	nanomolar
p	probability statistic
PDB	potato dextrose broth
PE	pectin esterase
pI	isoelectric point
pH	$-\log_{10}[H^+]$
PL	pectin lyase
RPHPLC	reversed phase HPLC
s	second
S	siemen

spp.	Species
visc.	viscosity assay
&	and
@	at
δ	change in
[ ]	concentration
°C	degrees centigrade
µg	microgram
µL	microlitre
µM	micromolar
µS	microsiemen
%	percent
®	registered
©	copyright

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INTRODUCTION

1.0 *Canadian Forest Productivity Trends*

Canadian forests comprise about 10% of the world's forested land and produce 14% of the world's coniferous harvest. Fully half of this comes from British Columbia where 51.2 million hectares are covered by productive forests (McLean 1994). Global wood consumption has increased from 1.5 billion m^3 to 3.5 billion m^3 of wood in the past 40 years, and we are presently near the maximum sustainable harvest level of natural forests (Sutton 1993). Higher productivity can only be supported by effective forest renewal. One of the most important factors impeding successful reforestation in recently harvested areas is the rapid establishment of opportunistic non-crop forest vegetation, such as deciduous hardwoods (Dorworth 1991). Integrated vegetation management (IVM), a concept encompassing all available forestry management tools, plays an essential role in current reforestation efforts (Stern *et al.* 1959, Stark 1977, Richardson 1993, Wagner 1993). In British Columbia alone it is estimated that there are greater than three million hectares of productive forest land that require some form of vegetation management (Boateng 1986). Control of hardwood regrowth is also important in industrial parks, shooting ranges, and the 69,000 km of existing BC Hydro rights-of-way.

Traditionally, competing hardwood vegetation has been controlled by manual cutting and/or by use of chemical herbicides to suppress the growth of forest weeds that compete with young conifers for light, nutrients and water. Over 90% of the chemical control of weeds in British Columbia is through the use of a single chemical, known as glyphosate (trade names: Round-up®, Vision®). Other chemicals, such as trichlopyr and imazypyr, are used less extensively (Dorworth 1991). The registration of new chemical herbicides for forestry is becoming more difficult, while some of the existing chemicals are being deregistered (Halleran 1990). Public perception is that herbicides can cause environmental damage and pose a hazard to people living in or near the forest, hence they are coming under closer scrutiny (Wagner 1993). Since most Canadian forests are on public land, the pressure to change vegetation management practices may increase, and possibly spread to private lands as the public continues to place greater value on natural forests, native animals and plants, and endangered species (Markin and Gardner 1993). This concern is well justified as many of the chemical herbicides in use today can affect non-target species, and some of the compounds may accumulate in the soil and water table with long term use (Strobel *et al.* 1992).

Manual control of vegetation is environmentally more desirable than chemical application but has serious drawbacks. Manual clearing of vegetation is labour intensive and expensive, and often results in vigorous regrowth of weed species through resprouting of stumps and rhizomes (Wall 1990, Oswald 1990, Wagner 1993). The regrowth of manually cleared deciduous species is of key importance in Canadian forestry efforts. Though beyond the scope of this study, germination of buried or windblown seed may compound the original weed infestation problem.

It is clear that as chemical herbicides and other potentially environmentally damaging vegetation management tools are phased out, new tools need to be developed to improve forest growth in an economically sustainable manner. Many of the new tools under consideration are labeled biological controls, or biocontrols.

2.0 ***Biological Controls in Forestry Vegetation Management***

The earliest form of agricultural biocontrol involved the manipulation of environmental conditions in order to induce pathogenicity or stimulate virulence of native micro-organisms towards the target species. The modern definition of biocontrol is "the deliberate use of natural enemies of a species in order to suppress the growth or reduce the population of that species" (Markin and Gardner 1993). This involves the direct application of living agents or their metabolites to the target to reduce its population. This definition can be further categorized into two other areas: the *classical* approach, and the *inundative* (bioherbicide) approach (Hasan and Ayers 1990, Wall *et al.* 1992).

The classical approach involves the importation and release of an exotic organism (e.g., viruses, fungi, insects) to control an introduced weed or pest species. An example of classical biocontrol is the control of skeleton weed (*Chondrilla juncea*) in the wheat belt of Australia by the introduced rust, *Puccinia chondrillina* (Templeton *et al.* 1979, Hasan and Ayers 1990). There are certain risks involved in the approach. With the release of an alien pathogen into a forest ecosystem, the risk to non-target native vegetation could be high. There would be a potential for epidemic outbreak of the exotic organism due to a lack of natural control influences. Moreover, most of the plants considered to be weeds in Canadian forests are native rather than introduced species, and many of these play important roles in the forest ecosystem or in the economy. Hence the classical approach would probably be of limited use (Wall *et al.* 1992, Markin and Gardner 1993, Wagner 1993). Consequently, the development of a native pathogen by inundative biocontrol is preferred.

Inundative biocontrol uses mass inoculation of native pathogens to yield biocontrol. In agriculture, it has been demonstrated that a pest or weed can be controlled by culturing or rearing large numbers of pathogen propagules, and then releasing them in a localized area during a susceptible stage in the life cycle of the target pest (Van Dyke 1989, Hasan and Ayers 1990, Markin and Gardner 1993). This manipulation causes the population of the biocontrol organism to rise above endemic levels and overcome the natural constraints that normally limit the development of an epidemic in nature (TeBeest and Templeton 1985, Te Beest *et al.* 1992, Templeton *et al.* 1979). The result is an enhanced disease status that can be controlled by varying the specific conditions required for disease development.

This concept of inundative control, which is still relatively new, has been applied in agriculture with promise of considerable success. The use of inundative biocontrol to enhance the growth of desirable plant species in forest vegetation management (specifically conifer species) and maintain right-of-way clearance is being developed (Halleran 1990, Markin and Gardener 1993, Wagner 1993, Wall 1996). The concept of biocontrol of forest weeds is appealing because it would reduce reliance on chemical herbicide use in Canadian IVM.

One of the most important considerations in the selection of a candidate biocontrol organism is the nature of the target weed. While the introduction of livestock and insects has been successful for biocontrol of certain agricultural weeds, they are unsuitable for control of forest weeds primarily because there are more than one species of forest weed in a given area (Markin and Gardner 1993). In order to develop a single specific tool to control forest weeds (such as alder, maple and poplar spp.), the candidate organism must be capable of suppressing the growth of most, if not all, woody deciduous species, while at the same time not harming the conifer species.

Fungi are causal agents of many plant diseases, a characteristic that makes them attractive as candidate biocontrol agents (Templeton and Heiny 1989). Plant pathogenic fungi are common in forest ecosystems and some are quite selective towards weeds for which biocontrol is needed. Additionally, control can be tailored so that nearby untreated crop trees (even of the same species) are not affected, allowing for selective application and operator control. A fungus that is being used to effect biocontrol has been termed a *mycoherbicide* (Templeton *et al.* 1979).

3.0 *Mycoherbicides and Their Potential Role in Forestry*

The use of fungi for biocontrol of unwanted vegetation has gained widespread acceptance (Wilson 1969, Mortensen 1986, Templeton 1986, Wall 1995). Fungi have recently been used in the operational control of various agricultural weed species in Canada and the United States (Table 1), but there are currently no mycoherbicides registered for use in forestry anywhere in the world (Charudattan 1985, Markin and Gardner 1993, Wall *et al.* 1992, Watson 1989).

Constraints placed upon the development of a mycoherbicide for forestry are different than those for agriculture. Agricultural weeds invade a monoculture, whereas most forest weeds are native pioneer species, which normally colonize a site after a major disturbance. Growing for many years, these weedy plants play a role in forest ecosystem succession. Since phytopathogenic fungi have evolved with their hosts, they suppress their host weed species, thus playing a part in succession. Thus, successful pathogens

Table 1. Currently registered mycoherbicide biocontrol agents for use in agriculture.

Trade name	*Controlling organism*	*Target species*	*Country*
DeVine[a]	*Phytopthora palmivora*	Strangler vine	USA
Collego[b]	*Colletotrichum gleosporoides*	Northern joint-vetch	USA
Casst[c]	*Alternaria cassiae*	Sicklepod, senna	USA
Velgo[d]	*Colletotrichum coccodes*	Velvetleaf	USA
Biomal[e]	*Colletotrichum gleosporoides*	Round leaf mallow	Canada

a Ridings 1986, Templeton and Heiny 1989, Auld 1990.
b Daniel *et al.* 1973, Templeton and Graavast 1984.
c Walker and Riley 1982.
d Wymore *et al.* 1987.
e Mortensen 1988, Auld 1990.

rarely cause host species eradication. Such agents, being biological by nature, permit the avoidance of adverse effects encountered with prolonged chemical herbicide use. Mycoherbicides thus have the potential to minimize adverse environmental side effects encountered with chemical use, and offer a safe and effective means for vegetation management for both agriculture and forestry (Markin and Gardner 1993).

From a practical standpoint, the candidate organism for development as a mycoherbicide must satisfy several criteria. It must grow rapidly on culture media, readily infect its target host under a variety of environmental conditions, and suppress growth or induce mortality of the target weed species in a relatively short time (Winder 1990, Wall *et al.* 1992). Effective control should be achieved through the inundation of a target with even a relatively weak pathogen (Templeton *et al.* 1979).

4.0 Chondrostereum purpureum *as a Mycoherbicide for Forestry*

The concept of using the fungus *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar in weed biocontrol was first advanced by Scheepens (1980) for the control of American black cherry (*Prunus serotina* Ehrh.), which was introduced into the Netherlands from North America circa 1920 to improve understory and litter in planted pine forests. It soon developed into a forest pest, by colonizing cleared areas and suppressing the growth of native species (de Jong *et al.* 1990). Successful inundative biocontrol experiments using local Netherlands *C. purpureum* isolates were established to control these weeds in Dutch forests.

The initial development of *C. purpureum* as a biocontrol agent to suppress regrowth of a wide variety of hardwoods in Canada began in the Maritimes with inoculation trials by Dr. R. E. Wall of the Canadian Forest Service (Wall 1986, 1990, 1991). The observation that *C. purpureum* was not host specific and could infect a variety of tree species was based upon studies dating back to 1907, in which mycelial inoculum was placed on fresh cut wounds of various hardwood tree species (Bishop 1979). *C. purpureum* has also been shown to be the causal agent of silver leaf disease in orchard crops, with reports of disease in areas such as New Zealand, South Africa, Europe, Canada, and the United States (Brooks and Moore 1926). While this fungus does infect many forest hardwood species, it does not infect conifer species, making it attractive for conifer plantation management (Wall 1990, Wall *et al.* 1992).

This trait is favorable since there are many different forest weed species, which compete for resources needed by the slower growing conifer species. In B.C. alone, there are several fast growing weed genera that choke out young conifer stands, including *Alnus,* *Acer, Betula, Populus* and *Rubus* spp. (Wall *et al.* 1992). Most species competing with Canadian commercial forest trees can be classified by region of major impact (Table 2).

Since *C. purpureum* is a wound invasive white-rot basidiomycete, it can only enter its host through a fresh wound. This limits the natural spread of the fungus, requiring wounding and inundation of the host in order to be effective (de Jong *et al.* 1990, Wall 1991). The mycelium invades and kills the cambium of living hardwood stumps, causing wood discoloration and decay, and preventing the growth of adventitious shoots (Brooks and Moore 1926, Bishop 1979, Miyairi 1988, de Jong *et al.* 1990, Wall 1990, 1994). This is very important in stand management as it may be necessary to effect control in only part of a hardwood stand. Certain hardwood species, such as alder, are becoming increasingly important as a source of pulpwood, hence site-specific control is required (Dorworth 1991).

Since *C. purpureum* is indigenous to Canadian forests, there already exist natural controls for the fungus within the environment (Fritz 1954, de Jong *et al.* 1996). These controls prevent naturally existing fungus from remaining at greater than endemic levels, and also prevent it from spreading significantly from its area of application, thus *C. purpureum* can be treated as a 'naturally occuring microbial' (Wall *et al.* 1992, Wall 1994). Taken together these trends support *C. purpureum* as a potential candidate mycoherbicide biocontrol organism.

The experimental inoculation of yellow birch and red alder stumps through cork borer punctures and axe wound frills (partial girdling) suppressed wound healing and cambial recovery, caused elongate cankers, and increased the likelihood of tree mortality,

Table 2. Major species competing with commercial forest trees in Canada and forest regions where they are predominant[1].

Species	*Regions of major impact*
Acer macrphyllum Pursh. Bigleaf maple	Coastal British Columbia
Acer rubrum L. Red maple	Ontario, Quebec, Maritimes
Acer spicatum Lam. Mountain maple	Ontario, Quebec, Maritimes
Alnus incana (L.) Moench. Speckled alder	Transcontinental
Alnus rubra Bong. Red alder	Coastal British Columbia
Calamagrostis canadensis (Michx.). Bluejoint	Alberta, Interior B.C.
Corylus cornuta Marsh. Hazel	Interior B.C. to Quebec
Epilobium augustifolium L. Fireweed	Transcontinental
Gaultheria shallon Pursh. Salal	Coastal British Columbia
Kalmia angustifolia L. Sheep laurel	Maritimes, Newfoundland
Populus tremuloides Michx. Aspen, poplar	Transcontinental
Prunus pensylvanica L. f. Pin cherry	Ontario, Quebec, Maritimes
Pteridium aquilinum (L.) Kuhn. Bracken fern	Transcontinental
Ribes spp. Currants and gooseberries	B.C., Ont., Que., Maritimes
Rubus idaeus L. Raspberry	Ontario, Quebec, Maritimes
Rubus parviflorus Nutt. Thimbleberry	British Columbia
Rubus spectabilis Pursh. Salmonberry	Coastal British Columbia
Salix spp. Willows	Transcontinental
Sambucus spp. Elderberries	Transcontinental

[1]From Wall *et al.* (1992)

suggesting that cutting and treating hardwood stumps with *C. purpureum* is a more effective control than cutting or girdling alone (Wall 1986, 1990, 1991, 1994). The increased mortality and prevention of resprouting associated with the fungal application suggested its potential of becoming an effective mycoherbicide for use in IVM.

Using *C. purpureum* as a biocontrol agent for forestry may stimulate fears of a subsequent increase in silver leaf disease epidemics in orchard crops located near an area of application, especially after pruning. Risk analysis studies indicate, however, that the relatively short-lived basidiospores produced by the fungus are unlikely to pose a risk to non-target trees (i.e., pruned fruit trees). Simulated basidiospore dispersal with a gaussian plume model estimated there would be 160 basidiospores/m^3 of air at 500 m distance from the emission point, but this drops to only 4 basidiospores/m^3 at a distance of 5,000 m (de Jong *et al.* 1990). Hence, a 500 m buffer zone is recommended, implying a risk of the same magnitude as naturally occurring spores. Furthermore, recent population genetics studies (utilizing RFLP and SCAR analyses) indicate that high virulence inoculum is rapidly dissipated by the local population.

5.0 *Phytotoxic Metabolites of* **Chondrostereum purpureum**

While *C. purpureum* is the primary causal organism of silver leaf disease, many broad-leaf deciduous trees can be infected by the fungus without exhibiting silver leaf symptoms (Setcliff and Wade 1973). The silvering of the foliage seen with fungal infection is caused by the partial physical separation of palisade cells from the epidermal layer, as well as separation of the cuticle from the epidermis (Brooks and Moore 1926, Miyairi *et al.* 1977). The resulting air pockets reflect incident light giving the leaves a silvery sheen. Brooks and Brenchley (1931) demonstrated that injection of sterile filtered water extracts from cultures of *C. purpureum* into host trees (either apple or cherry) produced the onset of silver leaf symptoms, and Naef-Roth *et al.* (1961) suggested that *C. purpureum* induced silver leaf symptoms through the actions of a secreted phytotoxin capable of digesting plant structures.

In 1977, Miyairi *et al.* determined that the causative agent of silver leaf disease was a toxic protein fraction produced by *C. purpureum* in the xylem of the infected tree and translocated to the leaves. This pectic enzyme fraction, which contains primarily endopolygalacturonase [poly-(1,4-α-D-galacturonide) glycanohydrolase EC 3.2.1.12] (endoPG) activity, can induce silver leaf symptoms in 2 - 3 year old apple trees when as little as 25 μg are injected per tree. Later, Miyairi *et al.* (1979) found that there are four endoPG isozymes present within the silver leaf inducing fraction, each with different isoelectric points. All isoforms induce silver leaf symptoms in young apple trees. The toxic protein fraction was also found to digest plant cell wall polymers, and was hence linked to the silver leaf symptoms observed in the separated epidermal and palisade tissues of affected foliage.

Miyairi and colleagues (1977) found that endoPG from *C. purpureum* is enzymatically similar with respect to optimum pH, thermal stability, and molecular weight to endoPG of *Lycopersicon* (tomato) and *Saccharomyces fragilis* (yeast), but that endoPG preparations from these species do not result in silver leaf symptoms when injected into apple trees. The endoPG of *C. purpureum* also shares physiological characteristics (such as mass, pH stability and thermal stability) with the fungi *Phanerochaete chrysosporium*, *Cryphonectria parasitica*, and *Saccharomyces cerevisiae* (Shanley *et al.* 1993, Gao and Shain 1994, Blanco *et al.* 1994).

Of the four endoPGs produced by *C. purpureum*, the major form present is the one with the highest isoelectric point (pI 8.5) and is referred to as endoPG I (Miyairi *et al.* 1985). This enzyme also demonstrates the highest specific activity, highest thermal stability (70°C for 30 minutes), and broadest pH stability (~pH 4 to ~pH 9). Miyairi *et al.* (1985) concluded that because endoPG I is stable over a broad temperature and pH range, that the enzyme could be expected to remain unaltered in a tree during the infection process. These authors concluded that endoPG I was one of the major phytotoxic factors produced by *C. purpureum*. Furthermore, a recent publication reports three glycosilated isoforms of endo PG I (masses: 42, 45, and 48 kDa) which share the same isoelectric point. From nucleotide sequence analysis of a 1212-bp open reading frame cDNA encoding 403 amino acid residues, the deglycosylated mass was calculated to be 39.1 kDa (Miyairi *et al.* 1997).

In addition to endoPG isoforms, the pectolytic fraction produced by *C. purpureum* contains exopolygalacturonases, pectin lyases, pectate lyases, and pectin esterases (Miyairi *et al.* 1985, Neate *et al.* 1988). According to Miyairi (1988), these other enzymes do not induce silver leaf symptoms on their own, nor do they act synergistically with endoPG to give onset of symptoms. Consequently, it was concluded the above four enzyme types are not significantly involved in the phytotoxicity of silver leaf disease, though they may be involved in the pathogenic process of *C. purpureum* (e.g., suppressed wound healing, vascular browning, and killing of lateral shoots).

Since pathogenicity is a multigenic trait, it is not surprising that *C. purpureum* produces other toxic metabolites that are not necessarily proteinaceous in nature, as has been found with many other phytopathogenic fungi (Abbas *et al.* 1991, Buchwaldt and Jensen 1991, Pena-Rodriguez *et al.* 1988, Stierle *et al.* 1988, 1993, Vesonder *et al.* 1992, Xiao *et al.* 1991). Additionally, Brooks and Brenchley (1931) described an extremely heat-stable extract that produced mild brown nectrotic lesions when applied to apple trees. This result was confirmed by Bishop (1979) using whole leaves which were exposed to ethyl acetate extracts of culture filtrates. Extracts from different isolates gave interveinal discoloration at varying levels, which was accredited to the presence of an unknown toxin.

Recently, Struntz *et al.* (1997) assayed a group of sesquiterpeniod *C. purpureum* metabolites (previously described by Ayer *et al.* 1984) known to possess dicot phytotoxicity. It was found that sterpuric acid was effective at inducing aspen (*Populus deltoides* x *nigra*) cell mortality at concentrations as low as 10 ppm. The possible identification of other readily identifiable low mass toxic molecules from ethyl acetate extraction, as well as by other extraction methods, should not be ruled out (Manulis *et al.* 1984).

6.0 *Biorational Compounds*

Natural phytotoxins extracted from pathogens and used as herbicides are referred to as biorational compounds. The use of natural products as herbicides is attractive for two reasons. First, the chemicals are naturally occurring, thus biodegradation pathways already exist for the toxins *in vivo* (Cutler 1991). Secondly, they can be used as templates for chemical modification in order to modify selectivity and/or activity, without disrupting original biodegradable properties (Duke and Lydon 1993). There is need in vegetation management for more environmentally and toxicologically safe herbicides that are more efficacious and selective than the herbicides currently available (Duke and Lydon 1993). One possible source of these improved herbicides are natural products purified from phytopathogenic organisms.

A well known herbicide of natural origin is bialophos, a foliage herbicide that is effective against a wide range of weed species. Originally produced by *Streptomyces viridochromogenes*, the functional group for this biorational compound is an analog of glutamic acid, known as phosphinothricin, which disrupts amino acid synthesis (Jobidon

1991). Bialophos is currently marketed as a herbicide in Japan. Another herbicide of similar properties, glufosinate, is a synthetic derived from bialophos.

There are many obstacles associated with the development and registration of biorational compounds as herbicides. Toxins may be effective at low concentrations and relatively easy to purify or synthesize, but mammalian toxicity (as in the case of fumonisin B_1) may be unacceptably high (Abbas *et al.* 1991, Duke and Lydon 1993). Some toxins are produced at very low levels by an organism and/or are structurally complex, resulting in purifications, structural determinations and herbicide trials that are not cost effective (Duke and Lydon 1993).

If purification is cumbersome, it may be easier to chemically synthesize the compound in question, as was recently done by Struntz *et al.* (1997), than to purify it by extraction. Similarly, many natural products have biologically active functional groups that can be used as templates for synthesis. Chemical modification can also be used to alter a product's activity, while at the same time maintaining the desired biodegradable nature of the compound.

Chemical modification can also alter target species selectivity, which is an important consideration in the selection of a biorational compound. Species specific compounds can be used in direct application (such as is the case with curvulin and maculosin; see Table 3) (Strobel *et al.* 1992). Typically, compounds that are non-host selective, however, have better commercial prospects because they can be used in a broader range of applications, yielding control over a greater range of weed species while maintaining cost effectiveness (Duke and Lydon 1993). Most phytotoxic compounds can

Table 3. Phytotoxic metabolites produced by various fungi, showing chemical families, relative activities, and species selectivity.

toxin name	*producing genus*	*chemical family*	*activity (approx M)*	*species (Y/ specific N)*	*ref.*
Bipolaroxin	*Bipolaris*	eremophilane	µM (mM)	Y (N)	*a*
Gigantinone	*Dreschlera*	eremophilane	µM	N	*a*
Petasol	*Dreschlera*	eremophilane	µM	N	*a*
Zinniol	*Alternaria*	eremophilane	µM	N	*b*
Prehelminthosporol	*Bipolaris*	sesquiterpenoid	mM	N	*c*
Ophiobolin	*Bipolaris*	sesterterpenoids	µM	N	*d*
Curvulin	*Dreschlera*	cyclic polyketide	nM (µM)	Y (N)	*e*
Methyldiaporthin	*Dreschlera*	isocoumarin	nM	N	*f*
Dihydropyrenophorin	*Dreschlera*	coumarin	µM	N	*a*
Tryptophol	*Dreschlera*	indole alkaloid	µM (mM)	Y (N)	*g*
Dihydroexserohilone	*Exserohilum*	diketopiparazine	µM	N	*a*
Maculosin	*Alternaria*	diketopiparazine	µM (mM)	Y (N)	*h*
Sirodesmin	*Phoma*	epipolythiodioxopiparazine	µM	N	*i*
Alterosin I & II	*Alternaria*	perylenequinone	mM	N	*j*
Destruxin	*Alternaria*	cyclodepsipeptide	µM	Y	*k*
Marticin	*Fusarium*	naphthazarine	µM	N	*l*
Fumonisin (B$_1$)	*Fusarium*	alkylamine	µM	N	*m*
Mellein	*Phoma*	naphthazarine	µM	N	*n*
Hydroxymellein	*Tubakia*	naphthazarine	µM	N	*o*

a Strobel *et al.* 1992
b Stierle *et al.* 1993
c Pena-Rodriguez *et al.* 1988
d Xiao *et al.* 1991
e Kenfield *et al.* 1988
f Hallock *et al.* 1988
g Sugawara and Strobel 1987
h Stierle *et al.* 1988
I Pedras *et al.* 1990
j Stierle *et al.* 1989
k Buchwaldt and Jensen 1991
l Cutler 1991
m Abbas *et al.* 1991
n Parisi *et al.* 1993
o Venkatasubbaiah & Chilton 1992

be classified according to the basis of their chemical families, relative activities or species selectivity (as per Table 3). Elucidation of these compounds gives important information about the mechanism of pathogenicity for the controlling organism.

7.0 *Phytotoxins and Their Roles in Pathogenicity*

Fungi that are effective pathogens can serve as a source for the discovery of novel biorational products. Plant pathogens may produce several toxins that play an integral role in the pathogenicity of an organism (Stierle *et al.* 1989, Duke and Lydon 1993), but pathogenicity is not merely the production of toxins which allow the pathogen to suppress and invade its host. It is a finely tuned system of complementary physiological virulence and avirulence factors that are tightly inter-linked and responsive to environmental conditions (Shaner *et al.* 1992). The production of toxic compounds that are involved in this host/pathogen relationship are strictly regulated so that a particular level of suppression is achieved. Levels of toxin production need to be tightly regulated by the pathogen so that problems, such as autotoxicity, can be avoided (Duke and Lydon 1993).

It has been well established that different isolates of *C. purpureum* show varying levels of pathogenicity (Ekramoddoullah *et al.* 1993, Shamoun *et al.* 1991a, 1991b, Shamoun and Wall 1996, Wall 1990, 1993, 1994, 1996, Wall *et al.* 1996). It is also apparent that endoPG is a major factor in pathogenicity of *C. purpureum* (Miyairi 1977). Since pathogenicity is a multigenic trait, it is not very likely that this enzymatic toxin is the only contributing factor in *C. purpureum's* pathogenicity. Other factors such as secondary toxic metabolites (e.g., sterpuric acid, sturpurene) can also be important (Bishop 1979). Most pathogenic fungi have several virulence factors (Strobel *et al.* 1992), and it was hypothesized that, in combination with other factors, low mass toxins could contribute significantly to the pathogenicity of *C. purpureum* (Struntz *et al.* 1997) It is possible that more virulent isolates of *C. purpureum* produce higher levels of endoPG, toxic secondary metabolites, or both (depending on the genotypes of those isolates), though the overall significance of these factors in the pathogenic process is not known. If *C. purpureum* is to be developed as a mycoherbicide, it would be practical to select a single effective isolate to control a diverse local target weed population. Identification of this optimal isolate could follow from selection of the most effective pathogen by determining field virulence, or potentially by assessment of virulence factors (e.g., endoPG) responsible for increased isolate efficacy. Currently, there are no rapid measures of virulence that can be used to predict field performance (Wall *et al.* 1996). In conjunction with field and genetics studies, toxin activity assays may prove to be effective operational tools for prediction, controlled breeding, and post-release assessment of *C. purpureum* isolate virulence.

8.0 ***Summary and Research Objectives***

As research into the mode of action of *C. purpureum* has continued, the study of the molecular biology and chemistry of the fungus has shifted. With the use of a select few isolates in operational field trials, the reasons for studying phytotoxic metabolites have become of greater importance. In addition to the potential of biorational discovery, it is also necessary to catalogue toxic metabolites for registration of the fungus as a bioherbicide. For registration of a microbial pesticide in Canada, this research supplements data required for medical and environmental toxicology regulations. Through this research, detected phytotoxins can potentially be used to assess *C. purpureum* virulence. The main objectives of this research include:

8.1 *To detect and characterize biorational compounds.* Many plant pathogenic fungi, including *C. purpureum*, are known to produce specific molecular toxins which are either low mass, peptidic, or proteinaceous in nature. Assessment and characterization of these toxins is necessary for both potential elucidation of biorational herbicides, as well as for the registration of *C. purpureum* as a mycoherbicide. Assays were developed for the detection of phytotoxic activity. Low mass toxin activity and relative levels of pectin degrading enzyme activity were assessed for five isolates of the fungus (four Canadian, one New Zealand).

8.2 *To determine the importance of toxins in pathogenicity.* Pathogenicity has been established as a multigenic trait, suggesting that many toxin producing phenotypes exist. Current virulence assays are labour intensive and require long incubation times, hence newer assays to quickly measure virulence were developed. This will allow for fast detection of relative phytotoxin activity, which could then be further tested in greenhouse and field inoculation studies. Assessment of the relative levels of pectin degrading ability were correlated to green house virulence data and bioassay data for 12 isolates of *C. purpureum* in an attempt to associate virulence with phytotoxin activity.

MATERIALS AND METHODS

1.0 *Fungal culture and induction of toxin production*

Five heterokaryotic isolates of *Chondrostereum purpureum* were selected for initial screening of phytotoxic compounds from the Pacific Forestry Centre's culture collection. Cultures were maintained on malt agar plates, and 1 cm^2 plugs from these plates were used to inoculate the liquid cultures.

Liquid culture media containing 0.1% citrus pectin was prepared as outlined by Wahlstrom *et al.* (1991) and used to induce the production of pectic enzymes by *C. purpureum*. Upon inoculation, isolates were grown (submerged by daily gentle agitation) at 20°C for 14 days in 150 ml Erlenmeyer flasks containing 20 ml of the pectic media per flask.

Three types of liquid cultures were prepared for the testing of phytotoxic metabolites: Potato dextrose broth (PDB, Sigma), PDB with 2% host effusion, and simple 2% host effusion. Host effusion was obtained by adding 1 g of hybrid-poplar (clone 20-50, Scott Paper Co.) shoots (bark removed and cut into small 1 cm diameter sections) per 100 ml media. Potato dextrose broth cultures (at 24.0 g/L) were grown for 14 days at 20°C in 100 ml aliquots contained within 250 ml Erlenmeyer flasks. PDB cultures containing an additional 2% host effusion (wt/vol) were also grown under the same conditions in 100 ml aliquots for 14 days. The simple 2% host effusion cultures were grown under the same conditions as the other host effusion media. All of the media were sterilized by autoclaving at 121°C for 20 minutes (host tissues remaining). Daily manual shaking was carried out following inoculation to ensure an even distribution of mycelium.

Liquid cultures were harvested by vacuum filtration through Whatman #1 filter paper to remove mycelia and other debris. The culture fluids were then further clarified by centrifugation at 6000 g for 30 minutes. The culture supernatants were pooled for each isolate of *C. purpureum* and stored at -20°C.

2.0 *Bioassay of phytotoxic metabolites*

A leaf disk bioassay and an electrolyte leakage bioassay were employed for the detection of the various metabolites produced by *C. purpureum*. Young succulent leaves (3 weeks to 2 months post-emergence) were removed from one of three deciduous indicator species grown at the Pacific Forestry Centre, Victoria, B.C.: *Populus balsamifera* x *deltoides* (hybrid poplar, clone 20-50, Scott Paper Co., New Westminister, B.C.), *Acer macrophyllum* Pursh. (bigleaf maple), or *Alnus rubra* Bong. (red alder). Leaf tissues were surface sterilized in 10% bleach for 60 seconds, and rinsed twice in sterile water prior to use.

Two types of qualitative leaf bioassays were employed, depending on the volume of extract available. The method used by Venkatasubbaiah and Chilton (1992) was employed only when small volumes of culture extracts or sample preparations were available. Fresh healthy leaves were cut into 4 cm^2 squares with a sharp scalpel. The centre of each piece of leaf tissue was lightly wounded (surface scratched) with the cut-off bristles of a small paintbrush. Twenty μl of sample (dissolved in 0.5% MeOH in dH_2O, pH 6.0) were placed onto the wounded area of the leaf. Leaf tissues were kept in 9.0 cm petri plate moist chambers for 48-72 hours. Fractions or samples which produced a necrotic reaction were designated as phytotoxic. Both uninoculated culture filtrate extracts and 0.5% MeOH were used as controls.

The method developed by Bussey and Stevenson (1991) was followed when larger volumes of extracts or sample preparations were available. Leaf disks, 10 mm in diameter, were cut from healthy leaves using a cork borer and floated on 2 ml of test sample contained within a single well of a 24-well tissue culture plate. Lesion development was evaluated after 72 hours. Inoculated leaf disks were rated visually on the basis of lesion surface area, lesion darkness, and the presence/absence of chlorosis. Ratings were based on a quantitative 0 to 5 scale: 0 is healthy (no necrosis); 1 is light speckling (up to 10% perimeter necrosis); 2 is up to 25% leaf disk necrosis (perimeter); 3 is 26-50% necrosis; 4 is 50-75% necrosis; 5 is up to 100% necrosis (central exceptions).

A modification of the electrolyte leakage bioassay described by Parisi *et al* (1993) was used to quantify phytotoxic activity. This assay was employed for its potential ability to detect membrane destabilizing toxins, a common mode of action among phytopathogenic fungal metabolites. Leaf disks, 5 mm in diameter, were taken from healthy leaves with a cork borer and 50 mg of these disks placed in a tube containing 1 ml of the sample to be tested (either culture filtrate or an extract preparation resuspended in H_2O containing 0.5% MeOH). The leaf disks were exposed to this solution for 15 minutes at room temperature, rinsed with dH_2O, and leached in 10 ml distilled H_2O for 90 minutes. The conductivity of this latter solution was subsequently measured using a Radiometer Copenhagen CDM 83 conductivity meter. Leaf disks exposed to uninoculated culture filtrate were used as controls.

3.0 *Extraction of non-enzymatic, low mass phytotoxic metabolites*

Culture fluids of the three media types used for production of non-enzymatic phytotoxins were extracted by the method of Xiao *et al* (1991) using ethyl acetate (EtOAc) extraction. Prior to extraction, culture filtrates, pre-filtered through Whatman #1 filter paper to remove mycelia and other debris, were centrifuged at 6000 g for 1 hour to remove any small amounts of cell and wood debris that may have remained in the culture fluid.

For the EtOAc extraction, cell-free culture supernatants were acidified to pH 3.0 by the addition of 6.0 N HCl and extracted three times with equal volumes of EtOAc. The aqueous phase was discarded and the EtOAc fraction was evaporated to dryness in a rotary evaporator under vacuum at 35°C. After evaporation, phytotoxic activity was determined for the residue following reconstitution in 0.01 M Na acetate buffer (pH 5.0). The weight of extract varied, depending on the isolate of *C. purpureum* used and the culture medium in which it was grown.

For those fractions demonstrating phytotoxicity, the EtOAc extraction residue was further separated into phenolic and neutral fractions as described by Venkatasubbaiah *et al* (1992). The residue was partitioned between EtOAc and 0.4% aqueous sodium carbonate. The phenolic fraction was retrieved from the aqueous sodium carbonate by acidification (addition of 6.0 N HCl) to pH 3.0 and re-extraction with EtOAc. After evaporation of solvents, the phenolic fraction (as extracted by EtOAc) was assayed for phytotoxic activity and the neutral fraction was discarded.

For comparison of ethyl acetate and phenolic extraction profiles, reversed phase high performance liquid chromatography (RPHPLC) was carried out for the various steps on a Pharmacia FPLC system using a Superpack C-18 cartridge. Comparative profiles were obtained using a solvent system of water (solvent A) and 70% methanol (solvent B). A linear gradient starting from 0% to 100% solvent B over 40 minutes was executed. The post run reports for each of these HPLC runs are archived at the Pacific Forestry Centre.

4.0 *Analysis of Pectinase Activity*

Pectic media culture fluids were examined for relative secreted production of pectinases by several methods and were compared between the various isolates by the following protocols.

Culture filtrate relative pectinase activities were measured by a viscosity assay using a size 300 Cannon-Fenske routine viscometer (International Research Glassware, Kenilworth, New Jersey). For each sample the viscometer was charged with 1.44% sodium polypectate in 0.01 M citric acid-0.01 M dibasic potassium phosphate buffer (pH 4.5) and equilibrated at 30°C in a water bath. Culture filtrates were concentrated 10 fold using an Amicon ultrafiltration cell (model 8050) with YM10 membranes (m.w. cutoff @ 10KDa) for assays which were used to screen the five original isolates pectinase activities. Centrifuge clarified (5000g for 30 minutes) crude pectin culture filtrates were used for viscosity assays of pectinase activity for comparison of isolates used in greenhouse virulence assays. A 1 mL aliquot was added to the viscometer, and time readings were taken at 5 minute intervals for 40 minutes (as measured by a stopwatch). The viscometer was cleaned, recharged with polypectate buffer, and temperature equilibrated in preparation for a new sample aliquot. Control values, equivalent to 0 mg/ml pectinase activity, were determined using concentrated uninoculated media. A 1mg/ml standard endoPG (Sigma) was used to standardize the assay. Since time measurements all continually increased over the 40 minute assessment period for each of the different isolate samples, the change in elution time (δ time) was calculated using the final reading for each isolate (optimal stop time, as per Table 12), and a viscosity reading (centistokes) was calculated for each isolate based on these elution time changes. Viscosity was calculated by multiplying the efflux time (s) with the viscometer constant (centistokes/s = 0.26104 for this apparatus). Relative activity was based on the ratio of sample viscosity to that of the uninoculated control (0 mg/ml pectinase). Specific protocols and viscosity measurements followed the "Instructions for use of the Cannon-Fenske routine viscometer" (Braulke 1991). EndoPG (from *Rhizopus* sp., Sigma Chemical Co.) was also assayed at a concentration of 1mg/mL as a comparative standard.

Partial purification of endoPG was carried out through column chromatography. Total protein was concentrated from culture filtrates by acetone precipitation with two volumes of -70°C acetone. The cold precipitated protein was collected by centrifugation at 15,000 g for 30 minutes and the residual acetone was removed under a stream of air. Pellets were resuspended in 0.01 M sodium acetate buffer (pH 5.0) and clarified by centrifugation at 5000 g for 30 minutes. The supernatant was applied to a 70 x 2.5 cm Sephacryl S-100 gel permeation column chromatography pre-equilibrated with 0.01 M sodium acetate/acetic acid buffer (pH 5.0). Fractions (3 ml) were collected and assayed for both protein and endoPG activity.

Two colorimetric assays were employed for detection of pectin degrading enzymes of culture fluids and HPLC fractions: a cup-plate indicator media assay and a reducing sugar assay. The cup-plate method, developed by Dingle *et al* (1953) and later modified by Cleveland and McCormick (1987), assayed the presence of pectic enzymes through substrate degradation and stain quenching. Twenty ml aliquots of this media were poured into 9.0 cm petri dishes, and using a sterile cork borer (3 mm diameter) five wells were symmetrically cut into each poured plate. Twenty-five µl of test sample was placed directly into each well and the plates were incubated at 30°C for 24 hours. The plates were then stained for two hours with 0.05% ruthenium red to assay pectin degradation. The diameter of a clear halo, indicative of pectin degradation (measured in millimetres), was used to assess relative activity. This assay was used as a general screening test for pectin degrading enzymes during purification steps.

The reducing sugar assay, developed by Bernfeld (1955) and later modified by Luchsinger and Cornesky (1962), indirectly tested for pectic degrading enzymes by assaying for sugars produced by the digestion of a pectin substrate. The key chromagen, 3,5-dinitrosalicylic acid (DNSA), was used for the detection of reducing sugars. The DNSA reagent was prepared as follows: 0.5 g of DNSA was dissolved in 20 ml of 2.0 M NaOH and 50 ml distilled water (dH_2O) in a 100 ml volumetric flask. 30 g of sodium potassium tartrate was added and dissolved, and the volume brought up to 100 ml with dH_2O. A 500µL volume of test sample was incubated with an equal volume of 0.2% citrus pectin (pH 5.0) at 25°C for 24 hours. Four volumes (2.0 mL) of the DNSA reagent were added and incubated at 75°C for 5 minutes. Samples were cooled for 10 minutes on ice and the absorbance read at 500 nm. Depending on the degree of color intensity, which may have been too high to read accurately on the spectrophotometer, the samples were diluted with a volume of H_2O and readings taken. The DNSA assay was used for chromatographic detection of pectinases in the size exclusion HPLC fractions. Results were compared to a standardized pectinase HPLC curve and a molecular weight standard curve.

5.0 *Greenhouse Pathogenicity Data*

Greenhouse trials were established in the early spring months (April 1995) using 12 *C. purpureum* isolates collected from southern Vancouver Island. Red alder saplings were grown to a height of 1m in individual pots in the greenhouse. Saplings were cut to a 15cm high stump, and the stump surface was immediately covered with a clay-talc formulation (Wall *et al.* 1996) containing living mycelium of one of the *C. purpureum* isolates (see Table 13). Control stumps were treated with the formulation material excluding the mycelium. Twelve saplings were treated for each isolate tested. Treatments were randomized in 12 replicate blocks in the greenhouse. After three months (July 1995), the extent of visual stump discoloration (cm of die-back), as well as the number and maximum height of stump sprouts (measured in cm), was assessed. The means of the above measured values were compared using the student t test. The number of stems that died was expressed as stump mortality (%) for five separate occasions for each isolate (Table 13). The relative mortality for each isolate was compared to pectinase activity to determine whether a correlation existed between these two parameters.

6.0 *Calculations for Significant Correlations, Relative Activity Indexes, and Figures.*

6.1 *Significance of tabulated data.*

For all tabulated data points included in the tables (Tables 4 - 16, means only), significance was determined by the student t test. Sample sizes (n) are indicated with the table headings (see specific Table). The numbers in parentheses indicate variance (σ^{n-1}) for the means.

6.2 *Indexes for significant results.*

6.2.1 Individual point indexes of relative assay activity (specific for that experiment only) are derived from significant experimental means. These indexes indicate relative activity trends for a given experimental parameter when totals for a given parameter are expressed (Figures 1 - 5). The following example illustrates the calculation of the index for isolate 2065 with respect to host extract culture medium and poplar indicator species (0.9). Example from Table 4 as per Table 17: isolate 2065/poplar/host extract mean minus control/poplar/host extract mean = 2.5-1.6 = 0.9. Point indexes for a specific significant tabulated finding are not shown, and only tabulated cumulative indexes are given for a specific experimental parameter (Tables 17 & 18).

6.2.2 Cumulative indexes for a given set of significant means were calculated for a given Table by adding all point indexes for a given parameter to obtain a cumulative index for a given Table. Cumulative indexes were not tabulated and were determined from significant point indexes. The following example illustrates the calculation of the cumulative index for poplar susceptibility. Example from Table 4 as per Table 17: sum (Σ) of all point indexes; = 0.9 + 0.8 + 1.3 + 1.0 + 1.0 + 1.1 + 0.8 = 7.3. When this is repeated for each of the other indicator species (following from the previous example): alder = 4.4; maple = 7.0. The relative activity cumulative indexes for the above calculations were tabulated as a ratio (Table 17): poplar = 1.00, alder = 0.60, maple = 0.96.

6.2.3 All of the cumulative post assay indexes were treated by the same protocol. Thus, the indexes in Tables 17 & 18 were assigned based on only one experimental parameter (i.e., indicator tissue used, media used, or isolate used). Since indexes were calculated based only on the significant differences from the controls of a given experiment, indexes between experiments (as per Table number) of relative toxicity cannot be compared. For example, an index of 1.00 for crude filtrate leaf assay (Table 4) does not mean that relative assay activity is the same as for an index of 1.00 for ethyl acetate leaf assay (Table 6) even though a trend can be noted for a given indexed experimental parameter (such as high activity for isolate 2139). Figures illustrating these indexes were based on the tabulated series, or averages thereof (as noted in the individual figure headings), which can be compared as a relative effect for a given parameter (e.g., media type) among the different experiments.

6.3 *Correlation coefficient statistics of greenhouse mortality with pectinase activity.*

The greenhouse mortality data were compared to determine whether there was a correlation between pectinase secretion of specific isolates and increased mortality on red alder due to infection by that isolate. An activity curve of mortality versus time for each isolates' greenhouse mortality was calculated based on the steepest slope (m, where y=mx+b, calculated using linear regression) of the straight line region of the curve up to it's first plateau (Table 19, Figure 6). These slope values estimated an early representation of fungal virulence during sapling infection. The 12 slope values (mortality/time) were compared to the relative activities of pectinase (as per viscosity assay findings, Table 16) using analysis of variance. Pectinase activity was also correlated with preliminary mortality assessments (July 1995, early sapling infection) using analysis of variance. All calculations were carried out using Quattro Pro 5.0 (Borland Software Co., Scotts Valley, California).

RESULTS

1.0 *Phytotoxins and potential biorationals from* **Chondrostereum purpureum**

In order to assess the relative effect of low mass and proteinaceous toxins from *C. purpureum*, assays were developed and relative phytotoxin activities were assessed for five isolates of the fungus (2065, 2097, 2121, 2139, SL33). The main objective for this project was the discovery and characterization of biorational compounds from *C. purpureum*. The culture filtrates of 14 day old culture filtrates were screened for the presence of phytotoxins by assaying crude extracts, ethyl acetate extracts, and phenolic extracts with both an excised leaf disk assay and an electrolyte leakage assay. Pectinase activity of these same filtrates was assessed by viscosity assay and was compared with the leaf disk assays. Three media types were utilized in the analysis, and three indicator species (big leaf maple, red alder, and a hybrid poplar clone) were used in the two leaf tissue assays. Relative indexed activities for a given parameter (e.g., media type, indicator species type, or isolate phytotoxin production trends) were based on the number of observations sharing significance at $p<0.05$ as determined by the student t test.

1.1 *Low mass phytotoxins*

Crude culture filtrates were first assayed using the leaf disk assay to determine relative levels of phytotoxin production (Table 4). The poplar and maple leaf disks demonstrated a greater sensitivity to phytotoxic activity than did the alder tissues. The fungus, when grown in the host infusion media, appeared to produce more phytotoxic activity as demonstrated by the indexes (Table 17). General relative indexed phytotoxin activity allowed a ranking of isolates from the least aggressive isolate 2121 to SL33 to 2065 to 2097 to the most aggressive 2139 (Table 17). The leaf disk assay gave the best representation of phytotoxic activity as indicated by a greater number of observations which were significantly different from the controls.

The electrolyte leakage assay measured the conductivity of a solution resulting from the release of cell constituents into a low osmolarity leaching buffer. The purpose of this assay was to detect phytotoxic activity of lipid soluble toxins that affect cell structural integrity or membrane permeability as well as cell wall degrading enzyme products, giving a relative conductivity value (in Siemens) for electrolyte detection. The maple and poplar tissues gave the highest indexed response to phytotoxin activity (Table 17). A greater number of significant phytotoxic observations were observed for the host extract and the host extract + PDB cultures. These data suggested that toxin production was increased in the presence of a host infusion. Isolates 2139 and 2097 demonstrated the highest indexed activities while the other isolates produced no significant observations (Table 4). The electrolyte leakage assay did not provide as many observations which differed significantly from the controls (6 for Table 5) as was noted for the corresponding leaf disk assay (19 for Table 4), hence demonstrating a poorer assay sensitivity when compared to the leaf disk assay.

As a first step in low mass toxin elucidation, ethyl acetate extractions were utilized since they have historically been shown to extract a significant portion and wide variety of phytotoxic compounds from different culture media (Stierle *et al.*

1988, 1993, Stierle and Cardellina 1989, Hallock *et al.* 1988, Xiao *et al.* 1991, Venkatasubbaiah *et al.* 1992). Proteins and debris were removed from the culture fluid by Amicon ultra-filtration using a membrane with a molecular weight cut-off of 10kDa. The majority of the larger protein toxins, including the pectin degrading enzymes, were thus removed prior to ethyl acetate extraction. The acidified and ethyl acetate extracted culture filtrates were tested by the leaf disk assay (Table 6). Extracts were reconstituted at 1/10x, 1x, and 10x original filtrate concentrations. No significant activity was detected for the [1/10x] samples. The largest number of significant observations were associated with the [1x] extracts, thus representing indexed activity at the phytotoxin concentrations that were encountered in the culture fluids. As indexed, the alder leaf disks were the least susceptible to damage (Table 17). Again, the host infusion media types produced more significant observations of phytotoxicity than the plain PD broth. Phytotoxic activity for *C. purpureum* isolates 2139 and 2097 was observed but the other isolates showed no significant activity, indicating a loss of phytotoxicity due to ethyl acetate extraction. Comparison with the leaf disk assay results from the crude filtrates (Table 4) revealed fewer significant observations by the same assay, suggesting that ultra-filtration or ethyl acetate extraction had removed some factor that was responsible for the majority of crude culture filtrate phytotoxicity.

The ethyl acetate extracts of each of the culture filtrates were also assayed by electrolyte leakage (Table 7). Again, the poplar and maple appeared to be more susceptible to indexed phytotoxin activity than the alder (Table 17). Unlike the crude electrolyte leakage assay where the host effusion media showed greater toxicity, the indexed ethyl acetate extract electrolyte leakage results indicated lower toxicity of media containing host extracts, converse to what was previously observed. Due to fewer numbers of significant observations, the production of phytotoxins could not be specifically linked to any particular isolate by indexed activity, again limiting the resolving power of this assay for phytotoxin detection compared to the leaf disk assay results (Table 17).

Phenolic extracts were further purified from the ethyl acetate extracts using the method of Venkatasubbaiah and Chilton (1991). These phenolic extracts were also reconstituted to [1/10x], [1x], and [10x] the original culture concentrations and assayed using leaf disks (Table 8). As was seen for the ethyl acetate extracts, the [1/10x] phenolic extracts exhibited no detectable toxicity. Again, the poplar and maple leaf disks appeared to be more susceptible than the alder in this assay (Table 17). The host infused media again demonstrated more phytotoxic activity than the non-augmented media (Table 17). All of the control experiments for this assay demonstrated relatively high disease ratings (Table 8), suggesting that phenolic media extractives interfere with assay performance. The total number of significant observations that were different from the controls for the phenolic extracts (4 for Table 8) were less than those seen in the leaf disk assay experiments with the ethyl acetate extracts (6 for Table 6). Though the difference is minimal, this suggests that some previously ethyl acetate extracted activity was lost in the unassayed neutral fraction, and that the remainder was phenolic in nature. The decrease is small, however, suggesting that most of the detected activity was extracted by the first ethyl acetate extraction, as was noted in other studies.

The electrolyte leakage assay results for the phenolic extracts are summarized in Table 9. The [1x] and [10x] samples showed little difference in the number of significant observations, suggesting that at high concentrations some metabolites extracted from the media interfere with the assay, making host extractives indistinguishable from fungal extractives at this stage. Relative indexed phenolic activity was detected only in the maple tissues (Table 17). This was the first assay where only the host infusion media was associated with increased phenolic toxin production (Table 17).

1.2 *Pectinolytic activities for* **Chondrostereum purpureum** *isolates*

In order to test the role of pectin degrading enzymes in phytotoxic activity, five *C. purpureum* isolates were grown on a medium containing citrus pectin as the sole source of carbon, and the culture filtrates were assayed for phytotoxic activity. The leaf disk and electrolyte leakage assays were used to assess the leaf damaging effects of putative degradative enzymes produced by the various isolates of *C. purpureum* and to compare the extent of damage to the values obtained for the phenolic and ethyl acetate fractions. The relative pectinolytic activity for each of the five isolates was measured using a viscosity assay (Braulke 1991). This assay allowed for quantification of pectin degrading ability and pectinase production, in the absence of low mass toxins (since they were filtered out upon sample preparation), for each isolate of *C. purpureum*.

The crude pectin culture filtrates were assayed using leaf disks of red alder, poplar (hybrid clone [20-50]), and big leaf maple. The negative experimental control consisted of uninoculated pectic culture medium and the positive control was purified endoPG (Sigma) at 1 mg/ml and 10 mg/ml. As was observed for the ethyl acetate and phenolic assays, the maple leaf tissues were most sensitive (Table 17). Toxin activity was ranked according to leaf damage and increased for isolate 2121 to SL33 to 2065 to 2097 to 2139 (Table 10). This was the same ranking as was observed in the crude culture filtrate leaf disk assay (Table 4). Phytotoxic activity was therefore reproducible for a given isolate among both complex (PDB), infusion (host & hostPD), and induction (pectin) media.

The electrolyte leakage data for the pectic culture filtrates were determined (Table 11). The average overall disease ratings were higher for the maple and poplar than for the alder leaf tissues (Table 17). According to this assay, isolates 2139 and 2097 induced the greatest degree of electrolyte leakage, while 2065, SL33, and 2121 showed the least activity. This recapitulated the results observed for the leaf disk assays (Tables 4 & 10).

Pectinolytic activity of a large number of isolates was determined using a viscometer assay. The reduced viscosity (centistokes) of a pectin syrup buffer indicated increased relative pectinolytic activity (Table 12). These results complemented those obtained in the leaf disk assay for the pectic culture filtrates, and demonstrated an identical indexed trend of pectin degrading activity, increasing from isolate 2121 to SL33 to 2065 to 2097 to 2139 respectively (Tables 10, 12 & 17). Since low mass toxins were filtered out upon sample preparation and do not affect viscosity measurements, the majority of toxin activity was likely due to pectin degrading enzymes.

2.0 *Toxins and their role in* **Chondrostereum purpureum** *pathogenicity*

To determine if pectinases play an important role in phytotoxicity, and if pectinase production can be correlated to virulence, laboratory phytotoxin assays were compared to a greenhouse assay of virulence among twelve *C. purpureum* isolates collected from southern Vancouver Island. Measurements of die-back, height, sprouts per stump, and mortality for each isolate were compared to the uninoculated controls. Isolates were ranked by their ability to cause mortality to red alder seedlings. Isolates 2140, 4u-88 and 2139 were found to be the most effective at inhibiting resprouting while 2047, 2088 and 2123x were largely ineffective (Table 13). Uninoculated stumps were used as a standard for comparison and all twelve replicates showed vigorous resprouting. For purposes of ranking isolate virulence, mortality (%) was tabulated and graphed over a one year time assessment period (Table 13, Figure 6). It was evident that, although all isolates were pathogenic, virulence varied among isolates. From the graph of mortality versus time (Figure 6), slopes were calculated for each isolate prior to the first observed level off phase. This parameter was used to estimate preliminary virulence for each isolate, since leveling out of mortality after this time was thought to be due to host species (red alder) resistance to the fungus.

Three supplemental assays, including the leaf disk, electrolyte leakage and viscosity assays, were conducted with the same 12 isolates to help discern the importance of pectinases in *C. purpureum* phytotoxicity. Leaf disk assays were performed on small aliquots of crude pectin medium culture filtrates (Table 14). Even though low mass toxin production was associated with host infusion (section 1.1), the possibility that low mass metabolites were contributing to activity was not eliminated.

As indicated for both leaf disk species, the highest phytotoxic assay activity was primarily associated with the more pathogenic isolates 2140, 4u-88, and 2139, and decreased for the isolates of lower pathogenicity (Table 18). Again, as in section 1.0, it was observed that red alder leaf disks were generally less susceptible to phytotoxic effects than the maple leaf disks. From the leaf disk assays (Table 14), it appeared that culture filtrates contained pectinases at a concentration lower than the relative effect of 1mg/ml *Rhizopus* pectinase. The electrolyte leakage assay demonstrated high variation, corresponding to a decrease in the number of significant observations (Table 15). Increased electrolyte leakage activity was associated with the more virulent isolates. activity was detected for the isolates that were more virulent to red alder seedlings. The phytotoxic effects of pectin degrading enzymes were more accurately detected by the leaf disk assay than the electrolyte leakage assay (Table 18). From the previous section (1.0), it was noted that reproducible phytotoxicity trends appeared to be more strongly associated with pectinase production. This observation was also noted for the twelve greenhouse isolates, with the higher mortality isolates having the greatest phytotoxic activity.

If, as hypothesized, the secretion pectinase plays a highly significant role in *C. purpureum* virulence, then perhaps there would be correlation between virulence of the various isolates and pectinase activity (Table 16). Pectinolytic activity was assayed directly from crude pectin culture filtrates to avoid loss of sample from processes such as concentration and degradation. A statistical analysis of relative pectinase activity to the calculated slopes of mortality indicated a highly significant correlation (P-value of 0.0306, Table 19). It appeared that higher fungal virulence, as estimated by the red alder sapling slope mortality data, was associated with an increase in pectinase activity. Additionally, a P-value of 0.0096 for the statistical correlation of mortality (July 1995) with pectinase further implicated a strong association of virulence with pectinase production (Table 19).

Table 4. Leaf disk assay of crude culture filtrates. Mean leaf disk assay disease rating (see materials and methods, n=5, variances in parenthesis) after 72 hours. Significant differences from the controls (student t test @ $p<0.05$) were noted by an asterisk. The media utilized include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay, plus the controls which consisted of uninoculated media.

TABLE 4.

Isolate	Species	Host Extract	PD Broth	Host & PD Broth
Control	Poplar	1.6 (0.4)	1.4 (0.4)	1.6 (0.4)
	Alder	1.4 (0.4)	1.2 (0.4)	1.5 (0.3)
	Maple	1.8 (0.4)	1.4 (0.5)	1.7 (0.3)
2065	Poplar	*2.5 (0.4)	*2.2 (0.3)	2.2 (0.5)
	Alder	2.0 (0.6)	1.9 (0.4)	2.0 (0.4)
	Maple	2.1 (0.3)	2.0 (0.4)	*2.5 (0.4)
2097	Poplar	*2.9 (0.5)	2.0 (0.5)	*2.6 (0.5)
	Alder	*2.6 (0.5)	1.9 (0.4)	*2.2 (0.3)
	Maple	2.4 (0.4)	*2.5 (0.5)	*2.8 (0.4)
2121	Poplar	2.3 (0.4)	2.0 (0.4)	2.1 (0.4)
	Alder	2.0 (0.3)	2.0 (0.5)	1.8 (0.5)
	Maple	2.1 (0.5)	1.8 (0.4)	*2.6 (0.3)
2139	Poplar	*2.6 (0.3)	2.3 (0.6)	*2.5 (0.3)
	Alder	*2.6 (0.4)	*2.5 (0.4)	2.2 (0.4)
	Maple	*2.9 (0.6)	*2.4 (0.3)	*2.7 (0.3)
SL33	Poplar	*2.4 (0.3)	2.0 (0.4)	2.2 (0.4)
	Alder	2.2 (0.4)	*2.2 (0.4)	1.9 (0.4)
	Maple	2.1 (0.5)	2.0 (0.6)	2.2 (0.5)

Table 5. Electrolyte leakage assay of crude culture filtrates. Mean electrolyte leakage conductivities (siemens (μS), see materials and methods, n=4, variances in parenthesis). Significant differences from the controls (student t test @ p<0.05) are noted by an asterisk. The media utilized include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay, of which the controls included uninoculated media.

TABLE 5.

Isolate	Species	Host Extract	PD Broth	Host & PD Broth
Control	Poplar	16.3 (2.4)	13.1 (3.0)	18.1 (3.1)
	Alder	14.2 (3.1)	15.2 (2.9)	16.5 (2.7)
	Maple	15.3 (4.2)	13.2 (3.7)	17.3 (2.9)
2065	Poplar	19.1 (3.1)	15.3 (3.8)	25.2 (5.1)
	Alder	21.3 (3.9)	12.1 (3.0)	20.2 (3.1)
	Maple	22.3 (4.6)	16.9 (2.2)	22.1 (4.3)
2097	Poplar	*27.2 (4.8)	18.6 (3.2)	*30.1 (5.3)
	Alder	22.6 (5.4)	17.3 (4.8)	24.9 (4.3)
	Maple	*24.1 (2.7)	*25.1 (6.3)	22.3 (3.2)
2121	Poplar	19.6 (2.3)	16.1 (3.1)	20.3 (2.8)
	Alder	18.3 (3.0)	18.1 (4.0)	19.6 (4.0)
	Maple	18.2 (4.9)	19.1 (4.3)	23.1 (3.8)
2139	Poplar	23.9 (5.3)	24.2 (5.8)	25.6 (4.0)
	Alder	*24.3 (1.8)	18.3 (3.6)	23.7 (3.8)
	Maple	24.6 (4.5)	18.6 (3.0)	*29.6 (5.3)
SL33	Poplar	23.4 (3.7)	19.1 (4.2)	25.3 (4.6)
	Alder	16.3 (2.2)	17.1 (3.7)	27.6 (6.0)
	Maple	20.3 (4.1)	17.6 (3.3)	23.3 (3.2)

Table 6. Leaf disk assay of ethyl acetate extracts. Mean leaf disk assay disease ratings (see materials and methods, n=5, variances in parenthesis) for varying concentrations of reconstituted ethyl acetate extracts. Extracts were reconstituted at 1/10x, 1x, and 10x the concentrations encountered *in vitro*. Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. The media utilized include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar and maple. Five isolates of *C. purpureum* were tested in this assay, of which the controls included uninoculated media.

TABLE 6.

		Host Extract			*PD Broth*			*Host & PD Broth*		
Isolate	*Species*	*1/10x*	*1x*	*10x*	*1/10x*	*1x*	*10x*	*1/10x*	*1x*	
Control	Poplar	0.8 (0.4)	1.5 (0.4)	3.0 (0.6)	0.9 (0.4)	1.2 (0.5)	2.9 (0.7)	0.8 (0.4)	1.5 (0.5)	3
	Alder	1.0 (0.5)	1.6 (0.6)	2.6 (0.6)	0.9 (0.4)	1.3 (0.6)	2.7 (0.5)	1.0 (0.3)	1.6 (0.4)	3
	Maple	0.8 (0.4)	1.5 (0.5)	3.1 (0.4)	0.8 (0.4)	1.5 (0.4)	3.1 (0.5)	1.2 (0.4)	1.8 (0.4)	3
2065	Poplar	0.7 (0.4)	*2.5 (0.4)	3.1 (0.6)	1.0 (0.3)	1.6 (0.4)	3.2 (0.5)	0.9 (0.3)	2.0 (0.5)	*4
	Alder	0.8 (0.5)	1.4 (0.8)	2.9 (0.4)	0.8 (0.4)	1.2 (0.5)	2.6 (0.4)	0.9 (0.4)	1.9 (0.4)	3
	Maple	1.2 (0.6)	2.1 (0.7)	3.5 (0.6)	1.0 (0.4)	1.8 (0.5)	3.3 (0.6)	1.1 (0.5)	2.1 (0.5)	4
2097	Poplar	0.8 (0.5)	2.0 (0.5)	*4.2 (0.4)	1.1 (0.4)	2.1 (0.5)	3.6 (0.4)	1.0 (0.5)	2.3 (0.4)	4
	Alder	0.8 (0.4)	2.0 (0.5)	3.5 (0.5)	1.0 (0.5)	1.8 (0.5)	3.6 (0.8)	0.9 (0.4)	2.3 (0.6)	3
	Maple	1.2 (0.4)	*2.5 (0.4)	3.7 (0.7)	1.6 (0.6)	2.0 (0.4)	3.7 (0.6)	1.4 (0.4)	*2.9 (0.5)	4
2121	Poplar	0.7 (0.4)	1.7 (0.5)	3.6 (0.5)	0.9 (0.4)	1.4 (0.6)	3.1 (0.3)	1.0 (0.3)	1.9 (0.5)	4
	Alder	0.9 (0.6)	2.1 (0.4)	3.1 (0.6)	1.0 (0.4)	1.4 (0.4)	2.9 (0.7)	1.0 (0.4)	2.0 (0.4)	*4
	Maple	1.0 (0.4)	*2.6 (0.4)	3.1 (0.5)	1.2 (0.5)	1.9 (0.5)	3.3 (0.5)	1.1 (0.3)	2.2 (0.5)	3
2139	Poplar	1.0 (0.5)	*2.4 (0.4)	3.8 (0.4)	1.8 (0.7)	1.5 (0.4)	3.5 (0.6)	0.8 (0.5)	*2.8 (0.6)	4
	Alder	0.7 (0.4)	1.9 (0.5)	3.2 (0.7)	1.0 (0.4)	2.0 (0.4)	3.2 (0.5)	1.2 (0.3)	2.0 (0.5)	4
	Maple	1.1 (0.6)	2.0 (0.6)	*4.1 (0.4)	1.3 (0.4)	1.5 (0.6)	3.4 (0.6)	1.4 (0.6)	2.3 (0.4)	4
SL33	Poplar	0.7 (0.4)	1.9 (0.4)	3.2 (0.7)	1.2 (0.5)	1.2 (0.4)	3.1 (0.6)	0.9 (0.5)	2.2 (0.6)	4
	Alder	1.0 (0.5)	2.1 (0.5)	3.1 (0.7)	0.9 (0.4)	1.4 (0.5)	*3.8 (0.4)	0.8 (0.5)	2.1 (0.5)	3
	Maple	0.9 (0.4)	2.5 (0.3)	3.0 (0.7)	0.9 (0.4)	1.5 (0.5)	3.0 (0.6)	1.2 (0.4)	2.3 (0.4)	3

*. Significant difference ($p<0.05$, n=5) in disease rating from the control.

Table 7. Electrolyte leakage assay of ethyl acetate extracts. Mean electrolyte leakage assay conductivities (seimens (μS), see materials and methods, n=4, variance in parenthesis) for varying concentrations of reconstituted ethyl acetate extracts. Extracts were reconstituted at 1x and 10x the concentrations encountered *in vitro*. Significant differences from the controls (student T test @ p<0.05) are noted by an asterisk. The media utilized to obtain the extracts include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay, of which the controls included uninoculated media.

TABLE 7.

		Host Extract		*PD Broth*		*Host & PD Broth*	
Isolate	*Species*	*1x*	*10x*	*1x*	*10x*	*1x*	*10x*
Control	Poplar	16.1 (3.2)	24.0 (3.1)	11.3 (2.1)	24.3 (4.0)	14.6 (2.9)	29.3 (4.
	Alder	13.2 (2.6)	23.4 (4.1)	11.1 (2.6)	20.6 (4.1)	14.3 (2.6)	27.2 (5.
	Maple	14.0 (3.1)	24.1 (3.3)	10.8 (3.7)	24.0 (3.3)	16.1 (3.2)	31.9 (4.
2065	Poplar	16.3 (2.0)	25.3 (4.0)	17.0 (3.4)	28.4 (3.8)	19.3 (3.6)	34.3 (3.
	Alder	15.2 (3.3)	26.6 (5.3)	14.2 (3.0)	22.3 (3.8)	12.2 (5.0)	22.6 (5.
	Maple	18.3 (2.7)	28.0 (3.0)	15.2 (4.3)	29.3 (3.2)	18.2 (3.8)	35.2 (4.
2097	Poplar	23.3 (3.9)	31.6 (3.5)	15.2 (4.1)	*36.2 (7.0)	19.3 (3.3)	37.8 (6.
	Alder	17.1 (2.7)	27.4 (5.6)	15.3 (3.3)	24.3 (4.1)	18.6 (4.3)	32.2 (3.
	Maple	21.3 (3.4)	31.4 (4.6)	15.1 (3.8)	28.7 (5.6)	*27.3 (5.2)	36.3 (4.
2121	Poplar	15.7 (5.6)	26.3 (4.8)	14.6 (2.4)	24.1 (4.3)	19.8 (3.2)	30.7 (5.
	Alder	18.6 (4.3)	21.6 (4.0)	*18.6 (4.3)	17.6 (4.3)	16.2 (4.8)	33.4 (4.
	Maple	17.2 (2.1)	27.0 (2.9)	15.8 (3.6)	25.1 (3.0)	19.2 (3.9)	28.6 (5.
2139	Poplar	*24.0 (3.8)	29.8 (4.2)	16.3 (3.2)	32.6 (5.3)	24.6 (5.0)	*46.6 (8.
	Alder	17.0 (2.8)	27.7 (3.0)	14.1 (3.9)	25.2 (3.0)	19.8 (2.7)	33.7 (4.
	Maple	19.3 (3.4)	31.0 (6.3)	13.1 (2.9)	28.9 (4.8)	*28.1 (5.5)	36.2 (3.
SL33	Poplar	18.3 (3.9)	27.0 (5.9)	16.5 (3.6)	26.1 (5.0)	18.3 (4.8)	34.7 (4.
	Alder	16.3 (2.0)	26.9 (4.4)	13.2 (2.2)	24.8 (4.7)	17.3 (2.7)	31.6 (3.
	Maple	17.1 (3.9)	30.3 (5.8)	19.7 (4.6)	26.3 (5.6)	22.8 (5.4)	33.1 (4.

Table 8. Leaf disk assay of phenolic extracts. Mean leaf disk assay disease rating (see materials and methods, n=5, variance in parenthesis) for varying concentrations of reconstituted ethyl acetate extracts. Extracts were reconstituted at 1/10x, 1x, and 10x the concentrations encountered *in vitro*. Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. The media utilized to obtain the extracts include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay, of which the controls included uninoculated media.

TABLE 8.

			Host Extract			*PD Broth*			*Host & PD Broth*	
Isolate	*Species*	*1/10x*	*1x*	*10x*	*1/10x*	*1x*	*10x*	*1/10x*	*1x*	
Control	Poplar	0.8 (0.4)	1.4 (0.4)	2.7 (0.3)	1.2 (0.5)	1.2 (0.4)	2.9 (0.6)	0.9 (0.5)	1.7 (0.5)	3
	Alder	0.9 (0.4)	1.2 (0.6)	2.6 (0.4)	1.0 (0.5)	1.2 (0.4)	2.2 (0.7)	1.0 (0.3)	1.8 (0.7)	2
	Maple	1.2 (0.5)	1.5 (0.4)	2.8 (0.6)	1.1 (0.4)	1.4 (0.3)	2.9 (0.7)	1.1 (0.4)	1.5 (0.4)	3
2065	Poplar	0.9 (0.4)	*2.3 (0.3)	3.0 (0.7)	0.9 (0.4)	1.3 (0.5)	3.0 (0.5)	0.9 (0.3)	1.6 (0.7)	4
	Alder	0.7 (0.5)	1.9 (0.5)	2.2 (0.6)	1.0 (0.4)	1.4 (0.5)	2.4 (0.5)	0.9 (0.4)	2.0 (0.6)	3
	Maple	1.0 (0.4)	2.1 (0.5)	3.0 (0.5)	1.1 (0.5)	1.6 (0.6)	2.8 (0.7)	1.3 (0.5)	1.9 (0.5)	3
2097	Poplar	1.0 (0.5)	1.9 (0.6)	*3.8 (0.5)	1.2 (0.5)	1.7 (0.6)	3.5 (0.5)	1.2 (0.5)	2.0 (0.5)	4
	Alder	0.8 (0.5)	1.7 (0.4)	3.4 (0.6)	1.2 (0.6)	1.6 (0.4)	3.0 (0.6)	1.0 (0.4)	2.2 (0.5)	*4
	Maple	1.3 (0.3)	1.9 (0.6)	3.3 (0.6)	1.5 (0.6)	1.8 (0.5)	3.5 (0.5)	1.0 (0.4)	2.1 (0.7)	4
2121	Poplar	1.0 (0.4)	1.2 (0.4)	3.1 (0.5)	1.1 (0.5)	1.3 (0.6)	3.0 (0.6)	1.1 (0.4)	1.9 (0.4)	3
	Alder	1.0 (0.4)	1.3 (0.5)	2.7 (0.7)	0.9 (0.4)	1.5 (0.6)	2.7 (0.8)	0.9 (0.5)	1.7 (0.5)	3
	Maple	1.2 (0.4)	1.4 (0.5)	3.1 (0.7)	1.4 (0.6)	1.3 (0.5)	3.0 (0.4)	1.1 (0.4)	2.0 (0.5)	*4
2139	Poplar	1.0 (0.5)	1.9 (0.5)	*3.6 (0.3)	1.4 (0.6)	1.9 (0.5)	3.2 (0.7)	1.1 (0.4)	2.3 (0.5)	4
	Alder	1.1 (0.6)	1.7 (0.5)	3.1 (0.5)	1.0 (0.5)	1.7 (0.4)	2.9 (0.4)	0.8 (0.5)	2.1 (0.5)	3
	Maple	1.2 (0.5)	1.7 (0.5)	3.5 (0.6)	1.4 (0.5)	*2.2 (0.3)	3.2 (0.5)	1.5 (0.6)	*2.6 (0.4)	4
SL33	Poplar	1.0 (0.5)	1.3 (0.4)	3.4 (0.8)	1.0 (0.4)	1.7 (0.6)	3.2 (0.5)	0.9 (0.5)	1.8 (0.5)	3
	Alder	1.1 (0.5)	1.3 (0.5)	2.7 (0.7)	1.1 (0.4)	1.3 (0.6)	2.4 (0.5)	1.1 (0.4)	1.9 (0.5)	3
	Maple	1.1 (0.5)	1.6 (0.4)	3.3 (0.8)	1.1 (0.5)	1.6 (0.6)	3.0 (0.6)	1.1 (0.4)	*2.5 (0.4)	4

Table 9. Electrolyte leakage assay of phenolic extracts. Mean electrolyte leakage assay conductivities (seimens (µS), see materials and methods, n=4, variance in parenthesis) for varying concentrations of reconstituted ethyl acetate extracts. Extracts were reconstituted at 1x and 10x the concentrations encountered *in vitro*. Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. The media utilized to obtain the extracts include host extract, PDB, and PDB & host extract. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay, of which the controls included uninoculated media

TABLE 9.

		Host Extract		*PD Broth*		*Host & PD Broth*	
Isolate	*Species*	*1x*	*10x*	*1x*	*10x*	*1x*	*10x*
Control	Poplar	11.9 (3.7)	24.0 (3.9)	12.1 (3.4)	21.6 (3.7)	17.8 (3.0)	27.9 (2.3
	Alder	12.8 (2.9)	21.3 (4.1)	10.6 (3.9)	20.0 (2.5)	15.3 (2.8)	27.0 (3.1
	Maple	12.7 (3.3)	22.0 (4.9)	11.5 (4.1)	21.6 (3.3)	15.3 (1.9)	25.3 (3.8
2065	Poplar	19.4 (4.0)	26.3 (5.4)	13.5 (4.6)	24.3 (2.3)	16.4 (3.5)	30.4 (6.1
	Alder	16.3 (3.1)	25.2 (4.0)	14.2 (3.9)	18.5 (4.1)	16.2 (3.7)	27.6 (4.0
	Maple	15.2 (4.3)	26.9 (4.1)	15.9 (2.4)	23.7 (2.0)	*25.6 (6.0)	28.4 (1.6
2097	Poplar	22.3 (3.6)	28.8 (4.7)	16.5 (2.9)	27.3 (3.6)	21.4 (4.6)	32.8 (4.6
	Alder	17.2 (4.6)	25.4 (6.3)	13.5 (4.0)	24.6 (4.3)	18.3 (1.3)	32.9 (4.0
	Maple	16.8 (4.7)	30.0 (4.7)	14.9 (3.3)	20.4 (7.6)	21.3 (4.4)	32.1 (5.3
2121	Poplar	13.9 (3.0)	27.5 (4.6)	12.6 (4.0)	27.4 (5.1)	18.9 (3.8)	28.1 (3.4
	Alder	14.3 (5.2)	22.3 (5.0)	11.3 (3.6)	26.9 (4.3)	14.9 (3.0)	23.6 (5.2
	Maple	15.7 (3.9)	27.0 (5.7)	15.3 (2.9)	24.0 (5.0)	15.0 (4.6)	*35.6 (4.1
2139	Poplar	18.0 (4.9)	34.3 (4.1)	15.3 (3.0)	27.0 (5.3)	24.6 (3.7)	33.0 (3.6
	Alder	17.2 (3.1)	27.2 (5.2)	14.5 (4.1)	26.3 (3.3)	19.2 (2.8)	36.3 (7.3
	Maple	*23.4 (4.3)	*35.3 (6.1)	15.2 (4.6)	23.9 (4.4)	*19.3 (4.9)	30.9 (6.8
SL33	Poplar	17.3 (3.1)	26.8 (4.3)	14.2 (2.8)	29.0 (6.7)	20.4 (5.1)	30.9 (4.3
	Alder	15.6 (3.4)	27.1 (4.6)	13.0 (4.0)	24.7 (4.9)	15.3 (3.0)	32.4 (5.8
	Maple	18.6 (4.0)	25.9 (5.6)	13.8 (4.1)	22.4 (3.6)	17.9 (4.1)	*35.6 (4.3

Table 10. Leaf disk assay of crude pectin culture filtrates. Mean leaf disk assay disease rating (see materials and methods, n=5, variance in parenthesis). Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. Crude pectin medium culture filtrates were utilized directly. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay. EndoPG pectinase (Sigma) from *Rhizopus* spp. (1mg/ml and 10mg/ml) and uninoculated pectin media were included as controls.

TABLE 10.

isolate	*poplar*	*alder*	*maple*
control	1.4 (0.3)	1.2 (0.4)	1.4 (0.2)
2065	2.3 (0.3)*	2.3 (0.4)*	2.7 (0.4)*
2097	2.5 (0.4)*	2.6 (0.3)*	2.9 (0.5)*
2121	1.9 (0.3)	1.8 (0.3)	1.9 (0.2)*
2139	3.0 (0.6)*	2.7 (0.3)*	3.5 (0.4)*
SL33	1.9 (0.3)	1.9 (0.2)*	2.4 (0.4)*
1 mg/ml pectinase	3.9 (0.4)*	3.2 (0.5)*	4.2 (0.5)*
10 mg/ml pectinase	4.5 (0.3)*	4.2 (0.6)*	4.8 (0.7)*

Table 11. Electrolyte leakage assay of crude pectin culture filtrates. Mean electrolyte leakage assay conductivities (seimens (mS), see material and methods, n=4, variance in parenthesis). Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. Crude pectin medium culture filtrates were utilized directly. The deciduous test species include alder, poplar, and maple. Five isolates of *C. purpureum* were tested in this assay. EndoPG pectinase (Sigma) from *Rhizopus* spp. (1mg/ml and 10mg/ml) and uninoculated pectin media were included as controls.

TABLE 11.

isolate	*poplar*	*alder*	*maple*
control	13.6 (3.1)	14.7 (2.8)	16.2 (4.1)
2065	21.6 (4.2)	17.3 (3.5)	22.9 (4.6)
2097	23.5 (3.3)*	20.2 (5.1)	26.2 (6.5)
2121	15.1 (2.1)	17.0 (2.8)	19.4 (3.3)
2139	22.3 (4.1)*	24.5 (5.3)	31.7 (7.4)*
SL33	18.4 (4.6)	19.2 (3.2)	19.7 (3.0)
1 mg/ml pectinase	30.1 (7.7)*	30.9 (5.6)*	36.1 (8.7)*
10 mg/ml pectinase	49.6 (10.8)*	42.6 (8.2)*	56.2 (9.1)*

Table 12. Viscosity assay of crude pectin culture filtrates. Mean viscosity assay readings (elution time change and centistoke value, n=3, variance in parenthesis). Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. Crude pectin medium culture filtrates were utilized directly. Five isolates of *C. purpureum* were tested in this assay. EndoPG pectinase (Sigma) from *Rhizopus* spp. (1mg/ml) and uninoculated pectin media were included as controls.

TABLE 12.

measurement	control	2065	2097	2121	2139	SL33	pectinase 1 mg/ml
δ time (s)	0.0 (0.0)	0.37 (0.02)	0.39 (0.02)	0.19 (0.02)	0.48 (0.01)	0.26 (0.03)	1.05 (0.03)
centistokes	1.67 (0.02)	1.56* (0.02)	1.55* (0.02)	1.61* (0.02)	1.53* (0.01)	1.59* (0.03)	1.36* (0.03)

Table 13. Red alder seedling greenhouse pathogenicity data. Measurements of die-back (cm), mortality (%), height (cm), and sprouts per stump for red alder seedlings inoculated in the greenhouse April 1995 with *C. purpureum* (see materials and methods, $n=4$). Significant differences from the controls were noted by an asterisk (at $p<0.05$) or two asterisks (at $p<0.01$). 12 isolates of *C. purpureum* were tested, the controls being uninoculated formulation. Note that isolates in parentheses are homokaryons, whereas all others are heterokaryotic. Isolates are ranked based on July 1995 die-back measurements.

TABLE 13.

	July 1995			mortality				
	Die-back	*Height(%)*	*Sprouts/*	*Mortality (%) over a one year assessment.*				
Isolate	*(cm)*	*(cm)*	*stump*	*May 95*	*July 95*	*Oct 95*	*March 95*	*May 96*
2140	10.5**	29.6**	4.6*	0	41.7	50.0	50.0	50.0
4u-88	8.9**	29.1**	3.3**	0	50.0	58.3	58.3	66.7
2139	9.0**	28.2**	4.9*	0	36.4	72.7	81.8	90.9
(2128u)	8.9**	28.3**	5.3*	0	36.4	63.6	81.8	90.9
3x-8u	9.0**	34.6*	5.2*	0	18.2	36.4	72.7	100
2072	8.3**	43.8*	7.2	0	25.0	33.3	41.7	58.3
3x-4u	7.8**	31.5*	6.5	9.1	36.4	54.5	54.5	72.7
2150	6.8**	45.0*	7.3	0	16.7	33.3	50.0	58.3
2090	5.7**	44.4*	8.8	0	0	41.7	66.7	66.7
2047	5.3**	40.9*	7.3	0	8.3	50.0	66.7	83.3
(2088)	3.6**	69.9	9.3	0	0	0	9.1	9.1
(2123x)	2.7*	71.2	9.5	0	0	0	0	9.1
Control	0.6	84.4	8.6	0	0	0	0	0

Table 14. Leaf disk assay of greenhouse isolates. Mean leaf disk assay disease ratings for the 12 greenhouse tested *C. purpureum* isolates (see materials and methods, n=4, variance in parenthesis). Significant differences from the controls (student t test @ $p<0.05$ and $p<0.01$) were noted by one or two asterisks, respectively. Crude pectin medium culture filtrates were utilized directly. Alder and maple deciduous indicator species were used. The controls included uninoculated medium and endoPG (Sigma) from *Rhizopus* spp. (1mg/ml).

TABLE 14.

isolate	*maple*	*alder*
2140	3.2 (0.7)**	3.0 (0.5)**
4u-88	3.5 (0.3)**	2.7 (0.7)*
2139	2.8 (0.7)*	2.5 (0.5)*
2128u	1.8 (0.3)	1.5 (0.3)
3x-8u	1.5 (0.5)	1.8 (0.3)*
2072	1.8 (0.3)	1.5 (0.3)
3x-4u	1.3 (0.3)	1.5 (0.3)
2150	2.3 (0.5)*	1.8 (0.5)
2090	3.8 (0.7)**	2.3 (0.3)*
2047	1.8 (0.3)	1.8 (0.3)*
2088	1.3 (0.3)	1.5 (0.3)
2123x	3.8 (0.5)**	2.0 (0.3)*
Control	1.3 (0.3)	1.0 (0.3)
1 mg/ml pectinase	4.3 (0.5)**	3.8 (0.7)**

Table 15. Electrolyte leakage assay of greenhouse isolates. Mean electrolyte leakage assay conductivities (μS) for the 12 greenhouse tested *C. purpureum* isolates (see materials and methods, n=3, variance in parenthesis). Significant differences form the controls (student t test @ p<0.05) were noted by an asterisk. Crude pectin medium culture filtrates were utilized directly. Alder and maple deciduous indicator species were used. The controls included uninoculated pectin medium and endoPG (Sigma) from *Rhizopus* spp. (1mg/ml).

TABLE 15.

isolate	*maple*	*alder*
2140	33.0 (5.3)*	23.7 (4.2)
4u-88	30.2 (7.2)	24.5 (3.8)
2139	31.1 (5.2)	25.1 (3.2)*
2128u	27.8 (4.1)	20.6 (5.2)
3x-8u	26.1 (5.0)	23.2 (2.7)
2072	27.6 (3.6)	21.4 (3.9)
3x-4u	25.3 (6.2)	24.1 (3.3)
2150	26.6 (4.4)	22.0 (4.1)
2090	34.3 (5.3)*	20.8 (3.6)
2047	26.2 (3.9)	20.7 (4.0)
2088	26.3 (4.7)	23.0 (5.1)
2123x	30.2 (4.2)	21.4 (3.3)
Control	21.0 (5.9)	18.3 (2.8)
1 mg/ml pectinase	33.2 (5.8)	28.2 (4.9)*

Table 16. Viscosity assay of greenhouse isolates. Mean viscosity assay readings (elution time change and centistokes, see materials and methods, n=2, variance in parenthesis) for the 12 greenhouse tested *C. purpureum* isolates. Significant differences from the controls (student t test @ $p<0.05$) are noted by an asterisk. Crude pectin medium culture filtrates were utilized directly. The control included uninoculated medium. Isolates are ranked by order of July 1995 die-back (as per Table 13).

TABLE 16.

isolate	*δ time*	*centistokes*	*relative activity (%)*
2140	0.32*	1.68 (0.02)*	90
4u-88	0.25*	1.70 (0.02)*	70
2139	0.35*	1.67 (0.03)*	100
2128u	0.24*	1.71 (0.01)*	60
3x-8u	0.17	1.72 (0.02)	50
2072	0.18	1.72 (0.02)	50
3x-4u	0.14	1.73 (0.03)	40
2150	0.24*	1.71 (0.02)*	60
2090	0.21	1.72 (0.03)	50
2047	0.13	1.73 (0.03)	40
2088	0.17	1.72 (0.02)	50
2123x	0.11	1.74 (0.02)	30
Control	0.0	1.77 (0.03)	0

Table 17. Indexes of relative assay activity for section 1.0. The cumulative significant results for all experiments in section 1.0 were indexed (see materials and methods) and tabulated. Indexes for a given data table are tabulated with reference to starting medium, deciduous assay species, and isolates of greatest activity. Indexes are in order (as per representative table number) of section 1.0 experiments. LD, leaf disk assay. EL, electrolyte leakage assay. Visc., viscosity assay.

TABLE 17. Post assay indexes for all experiments in section 1.0 (1.1 and **1.2**).

Table	Assay	Type	Poplar	Alder	Maple	Host	PD	HstPD	2139	2097	2065	SL33	2121
4	LD	crude	1.00	0.60	0.96	1.00	0.78	0.97	1.00	0.90	0.36	0.26	0.13
5	EL	crude	0.69	0.30	1.00	1.00	0.40	0.82	1.00	0.52	0	0	0
6	LD	EtOAc	1.00	0.20	0.75	1.00	0.18	0.77	0.97	1.00	0.64	0.33	0.73
7	EL	EtOAc	1.00	0.30	0.92	0.20	0.50	1.00	1.00	0.90	0	0	0.29
8	LD	phenolic	0.73	0.33	1.00	0.58	0.16	1.00	1.00	0.86	0.32	0.36	0.39
9	EL	phenolic	0	0	1.00	0.59	0	1.00	1.00	0	0.58	0.37	0.37
10	LD	crude pectin	0.56	0.89	1.00	-	-	-	1.00	0.77	0.63	0.52	0.10
11	EL	crude pectin	1.00	0	0.83	-	-	-	1.00	0.41	0	0	0
12	visc.	crude pectin	-	-	-	-	-	-	1.00	0.86	0.79	0.57	0.43

Table 18. Indexes of activity for section 2.0. Cumulative significant results for all experiments in section 2.0 were indexed (see materials and methods) and tabulated. Indexes for a given table are tabulated with reference to deciduous indicator species, assay type, and isolates of greatest activity. Indexes are in order of section 2.0 experiments (as per representative table number).

TABLE 18. Post assay indexes for all experiments in section 2.0.

Table	Assay	Type	Maple	Alder	2140	4u88	2139	2128u	3x8u	2072	3x4u	2150	2090	2047	2088	2123x
13	GH	-	-	-	0.86	1.0	0.79	0.62	0.46	0.57	0.62	0.29	0.14	0.14	0.00	0.00
14	LD	crude	1.00	0.78	1.00	1.00	0.75	0	0.21	0	0	0	0.97	0.21	0	0.90
15	EL	crude	1.00	0.26	0.90	0	0.51	0	0	0	0	0	1.00	0	0	0
16	visc.	crude	-	-	0.90	0.70	1.00	0.60	0	0	0	0.60	0	0	0	0

Table 19. Correlation coefficients for greenhouse mortality and pectinase activity. The relative activities of viscosity reduction (pectinase activity) and greenhouse mortality (slope) for each of the 12 southern Vancouver Island isolates were calculated from the first level off point of the mortality versus time results (Figure 6). A correlation to relative pectinase activity was carried out using ANOVA. A correlation to July 1995 mortality was also carried out with respect to relative pectinase activity. Values used in the calculations are tabulated, and calculations were carried out using Quattro Pro (Borland)

TABLE 19.

Isolate	Relative pectinase activity	July 95 mortality	Slope of mortality	P-value (pectinase vs. July95)	P-value (pectinase vs. slope)
control	0	0	0	0.030954	0.009592
2140	90	41.7	9.43		
4u88	70	50	11.68		
2139	100	36.4	6.78		
2128u	60	36.4	6.85		
3x8u	50	18.2	7.89		
2072	50	25	3.96		
3x4u	40	36.4	8.84		
2150	60	16.7	3.72		
2090	50	0	7.30		
2047	40	8.3	6.92		
2088	50	0	0.92		
2123x	30	0	0.54		

Figure 1. Indicator species combined average activity indexes. This illustration represents the combined average index for a given indicator species (poplar, alder, maple) for all isolates, media types, and assays. Numbers used in these calculations are included in Table 17 (section 1.0 experiments, Tables 4-11), and zero values are included in the calculation of the represented average.

Figure 1.

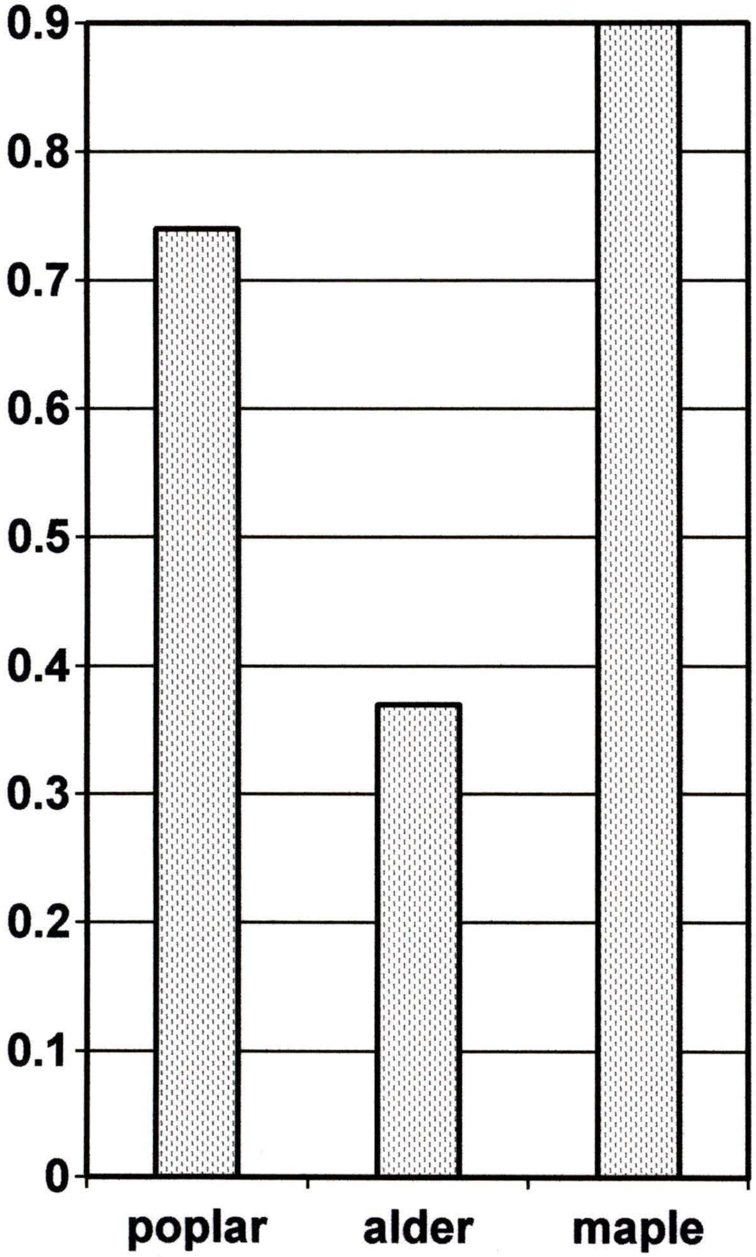


Figure 2. Medium composition combined average activity indexes. This illustration represents the combined average index for a given media type (host, PD, or host + PD) for all isolates, indicator species, and assays. Numbers used in these calculations are included in Table 17 (section 1.0 experiments, Tables 4-9), and zero values are included in the calculation of the represented average.

Figure 2.

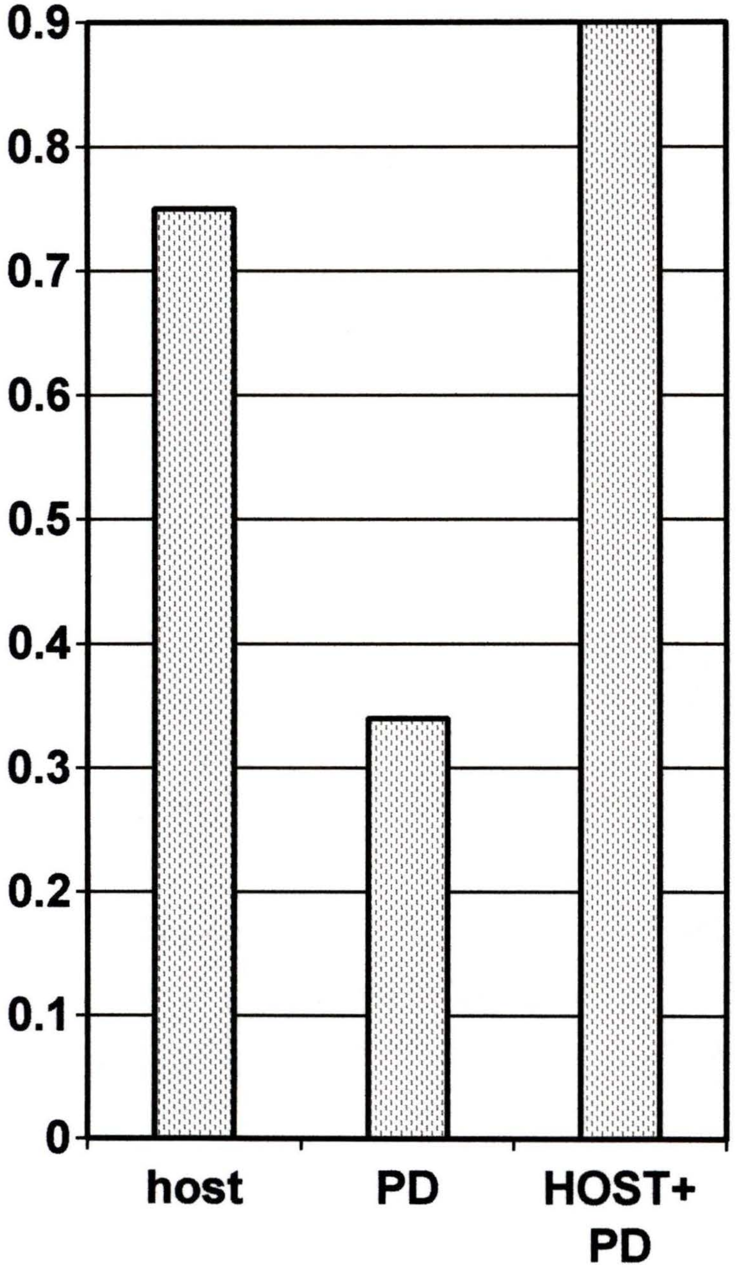


Figure 3. Culture fluid isolate toxicity combined average activity indexes. This illustration represents the combined average index for a given *C. purpureum* isolate (2065, 2097, 2121, 2139, or SL33) for all indicator species, media types, and assays. Numbers used in these calculations are included in Table 17 (section 1.0 experiments, Tables 4-12), and zero values are included in the calculation of the represented average.

Figure 3.

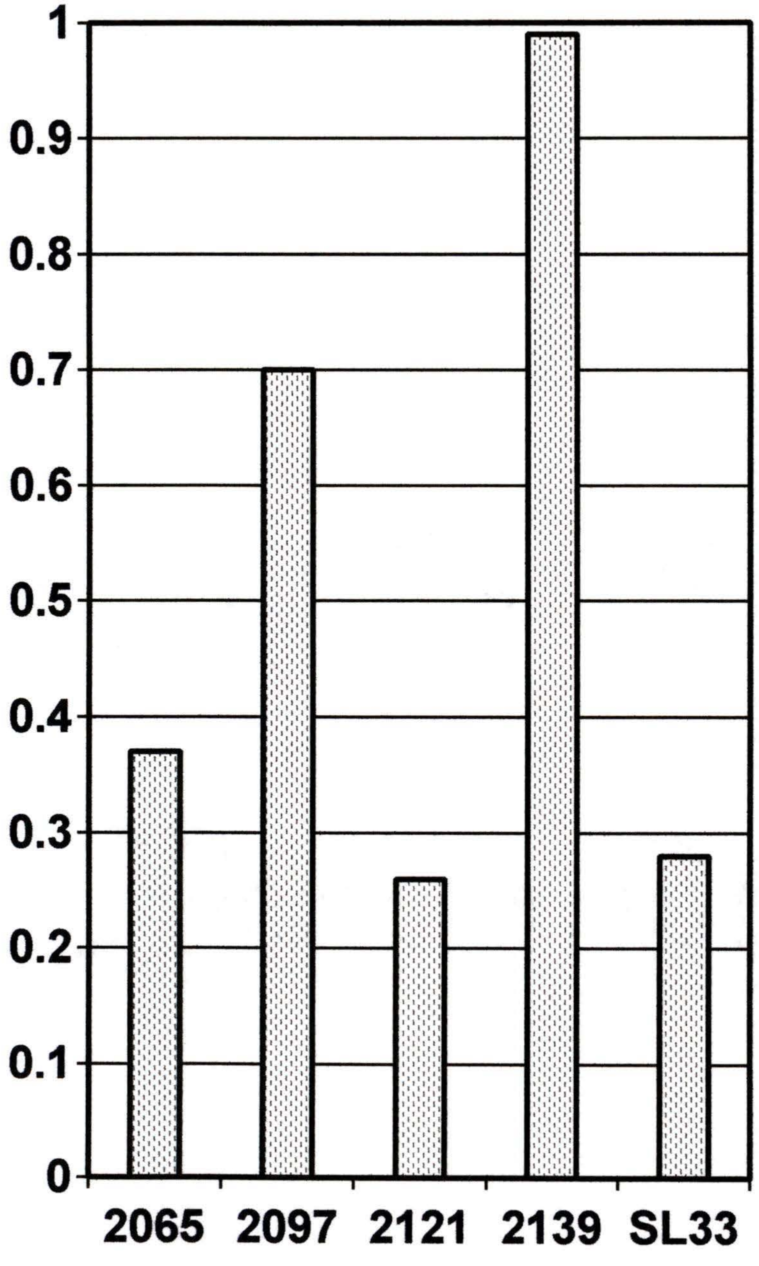


Figure 4. Crude leaf disk assay culture filtrate toxicity combined average activity indexes for each isolate of *C. purpureum*. This illustration represents the combined average index for a given isolate (2065, 2097, 2121, 2139, or SL33) for each of the crude media assay types. These are grouped as combined (host, PD, host+PD) and pectinase induction (pectin) medium types. Numbers used in these calculations are included in Table 17 (section 1.0 experiments, Tables 4,5,10 and 11), and zero values are included in the calculation of the represented average.

Figure 4.

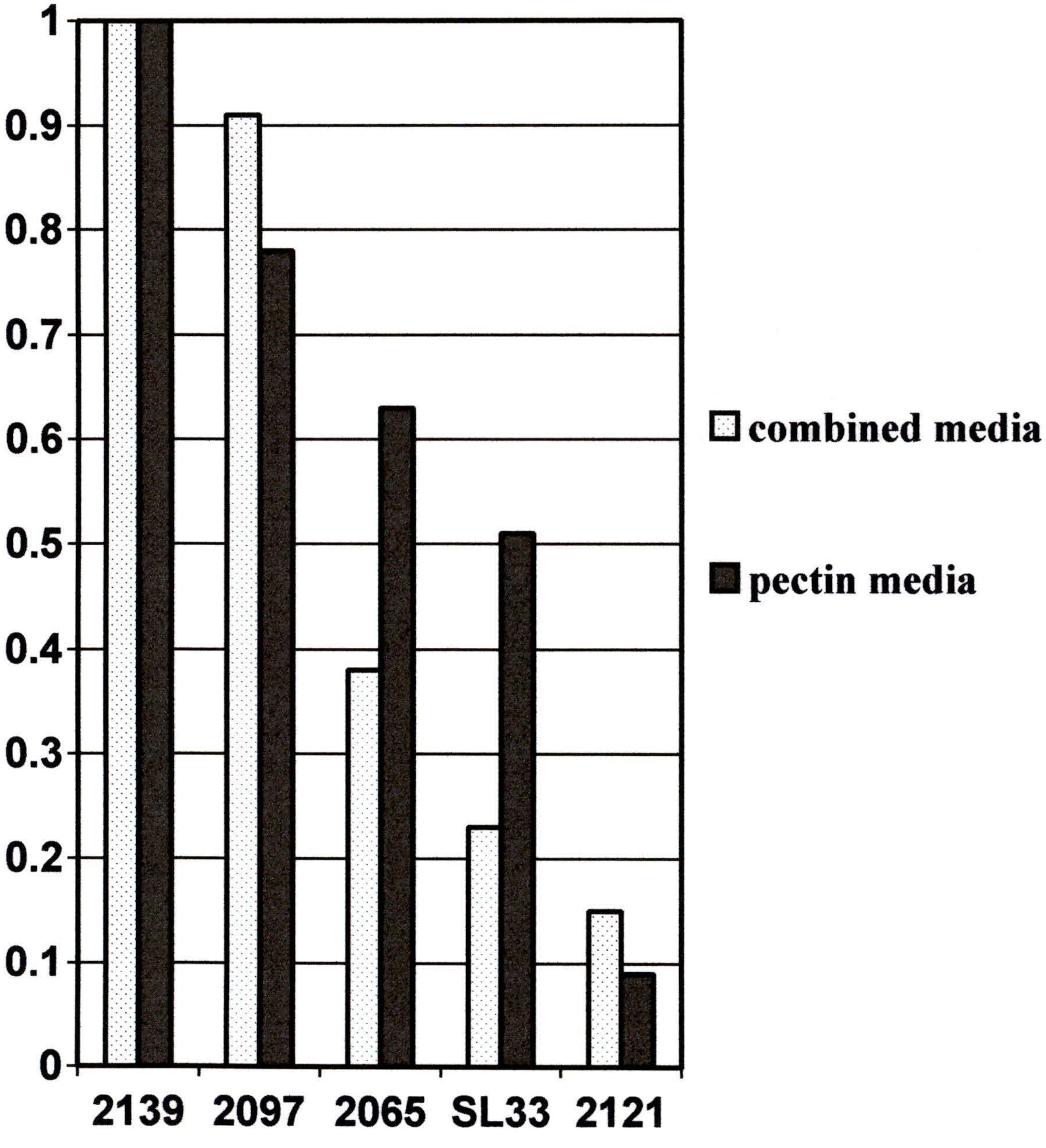

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2139
2097
2065
SL33
2121
☐ combined media
■ pectin media

Figure 5. Crude pectin viscosity assay relative activity indexes for each isolate of *C. purpureum*. This illustration represents the indexes for the viscosity assay of the crude pectin culture filtrates for each given isolate (2065, 2097, 2121, 2139, or SL33). Numbers used in this figure are included in Table 17 (section 1.0 experiments, Table 12). These trends are reproducible for a given isolate.

Figure 5.

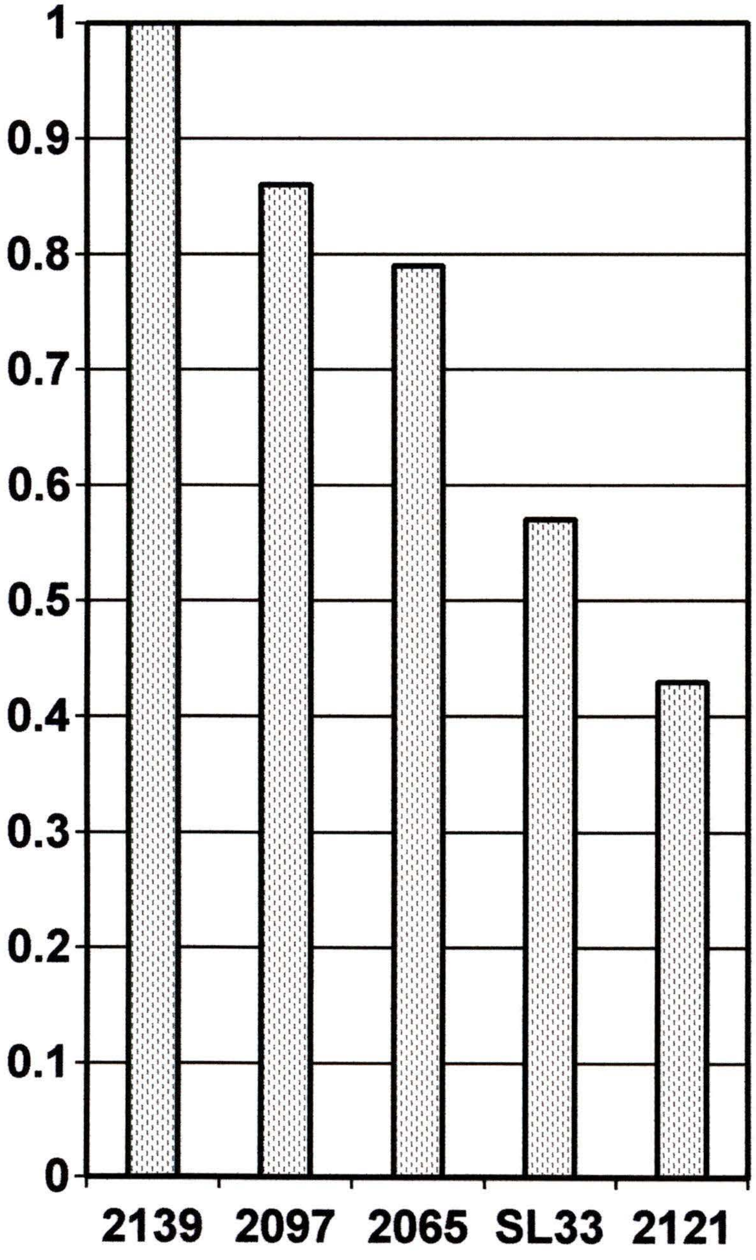
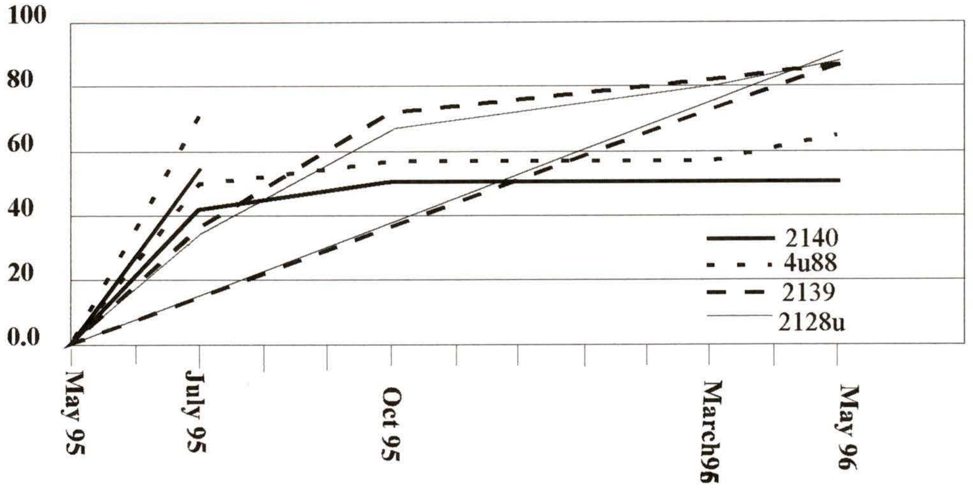


Figure 6. Mortality of red alder seedlings over a one year period. Mortality for the 12 greenhouse isolates was assessed over a 13 month period, as per Table 13, and plotted with mortality versus time. The isolates are presented in three groups of four, with the more virulent isolates being at the top, intermediate in the middle, and least at the bottom. The initial slopes of the mortality curves (up to and including the first plateau stage of the mortality assessments) were calculated for each isolate using linear regression (see materials and methods), and used in statistical correlation with pectinase activity. Representative slopes of mortality are given as a straight line in the graph, corresponding to the original isolates mortality. The mortality slopes are drawn to an extent representative of the months of high activity. For example, the slope of isolate 2140, as shown by the thick black line, extends to the July 95 date, corresponding to the first level off phase of 2140 mortality, with the linear regression analysis involving only the first three months of mortality for this isolate. Similarly, longer slope lines were calculated based on a larger number of mortality assessment dates. Mortality (%) is on the vertical and time (months) is on the horizontal.

Figure 6.


100
80
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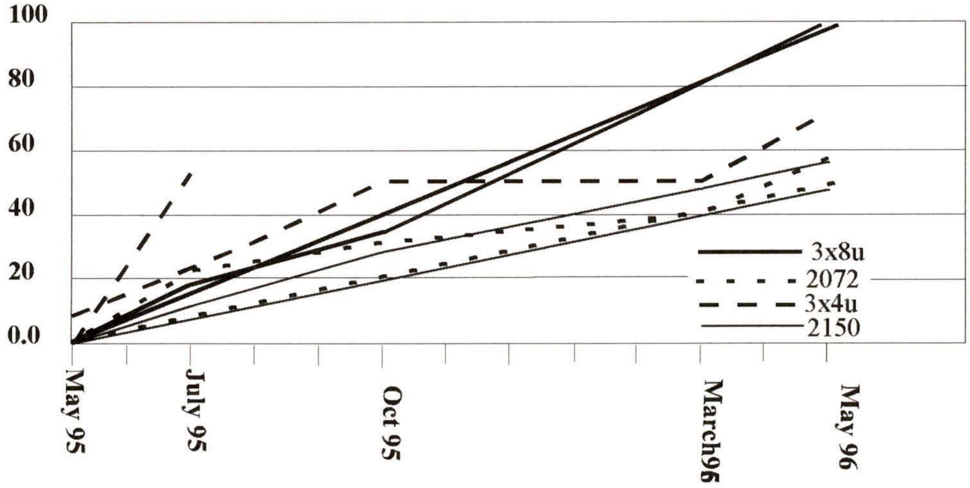
2140
4u88
2139
2128u
May 95
July 95
Oct 95
March96
May 96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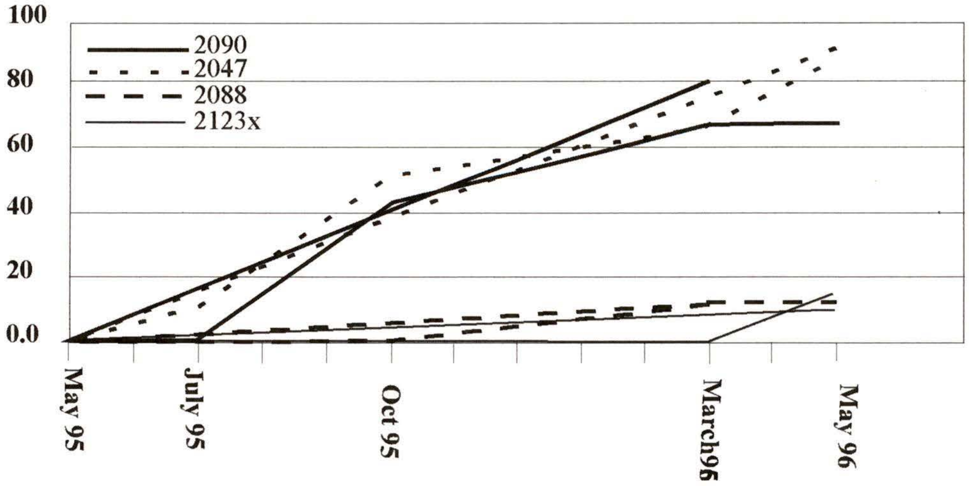
May 95
July 95
Oct 95
March96
May 96


100
80
60
40
20
0.0
2090
2047
2088
2123x
May 95
July 95
Oct 95
March96
May 96

DISCUSSION

1.0 *Phytotoxin detection and potential biorationals from* **Chondrostereum purpureum**

During the development of a mycoherbicide, the demonstration of phytotoxic metabolites by the biocontrol organism can be important for development of biorational compounds. Identification of these metabolites could help researchers understand the mode of action of pathogenesis by the fungus (Duke and Lydon 1993). Analyses of produced toxins are also needed for registration of bioherbicides. Phytotoxic metabolites produced in liquid culture were studied for five isolates of *Chondrostereum purpureum* in order to elucidate this biorational potential.

Both nutritive and environmental culture methods can have an influence on the production of secondary metabolites produced by fungi, hence toxin activity was assayed in crude culture filtrates derived from three different media. Defined media, where the relative amounts of defined salts, carbon, and nitrogen sources are known, is best for discrimination between compounds secreted by fungi and complex molecules from the medium itself (Strobel *et al.* 1992). Because of the close association between host and pathogen, it can often be difficult to find the nutritive and environmental conditions necessary for the growth of a given pathogen (Duke and Lydon 1993, Stierle *et al.* 1988, 1993). *Chondrostereum purpureum* grew well on many familiar media types, but like many basidiomycetes it required an additional organic source of nitrogen (Dhingra and Sinclair 1985). Many plant pathogenic fungi do not produce phytotoxic metabolites in a constitutive manner, thus making a host infusion medium necessary for inducing toxin production (Stierle *et al.* 1988, Strobel *et al.* 1992). During toxin purification studies, it is advisable to keep the levels of additives to a minimum, since mixed fungal metabolites and host extractives may only render the toxin purification process more difficult (Strobel *et al.* 1992, Pedras and Seguin-Swartz 1992). Indexed values of relative activity for the three media types utilized are given (Table 17). Across all extract preparations (i.e, crude, EtOAc, and phenolic) and assay types (leaf disk and electrolyte leakage) there was a stronger activity associated with host extract media. Addition of host infusion to potato dextrose broth also increased toxin production (Figure 2).

The leaf tissues used in both the leaf disk and electrolyte leakage assays revealed target species differences. The relative activity indexes (Table 17) demonstrated that phytotoxin activity was more readily detected by the maple and poplar leaf tissues than by the alder leaf tissues (Figure 1). I would suggest that these differences could be due to factors such as age of tissue, cuticle thickness, and other morphological differences among leaf species. The thicker cuticle of the alder tissues could to contribute to the observed phytotoxin resistance, and the larger surface area associated with the greater number of poplar and maple leaf disks may have resulted in increased phytotoxin surface exposure and penetration (Abbas and Boyette 1991, Brown and Hunger 1993, Venkatasubbaiah *et al.* 1991, 1992, 1993). This does not indicate species selectivity for *C. purpureum* phytotoxins, though the existence of species specific toxins is common among many plant pathogenic fungi.

The nature of toxin activity was suggested by the leaf disk and the electrolyte leakage assays (Tables 4 - 9). A greater number of observations significantly different form controls were observed for the leaf disk assay than for the electrolyte leakage assay (Table 17), thus more confidence was placed in the leaf disk assay as a quantitative assessment of phytotoxic activity. In support of this, the host infusion media did not appear to significantly increase phytotoxic activity for the phenolic electrolyte leakage data (Table 7). Electrolyte leakage readings that were encountered by this assay were in µS and much lower than normally encountered (typically mS) for many semi-purified membrane destabilizing toxins produced by ascomycetes and basidiomycetes (Abbas *et al.* 1992, Parisi *et al.* 1993, Pena-Rodriguez *et al.* 1988, Dhingra and Sinclair 1985). Thus the leaf disk assay gave more robust indications of phytotoxicity

1.1 *Low mass phytotoxin detection*

Unlike the electrolyte leakage assay, the leaf disk assay did not discriminate between phytotoxic modes of action. Activity was detected for all phytotoxins that could affect the foliage. Activity included both low mass toxins as well as proteinaceous toxins, such as cell wall degrading enzymes. The leaf disk assay of the crude culture filtrates (Table 4) indicated decreasing activity from isolates 2139 to 2097 to 2065 to SL33 to 2121 (Figure 3). Removal of proteins during extract preparation decreased the number of observations significantly different from the controls for culture filtrate phytotoxic activity, suggesting a link to large molecular weight toxins such as proteins. It has been established that cell wall degrading pectinases play an important role in *C. purpureum* phytotoxicity (Miyairi *et al.* 1977, 1979, 1985). Since both the ethyl acetate and phenolic extracts were protein free, it is suggested that some phytotoxic fraction (possibly proteinaceous in nature) was removed. This is based on the observed decrease in the number of results that were significantly different from the controls in Tables 6 & 8 relative to Table 4. The remainder of activity was apparently due to low molecular weight toxins.

It is apparent that low mass toxic activity existed in the ethyl acetate and phenolic fractions of the culture filtrates, confirming the findings of Bishop (1979) and establishing that low mass toxins could play some role in pathogenicity. Though low mass phytotoxin activity has been reported for some known low mass secondary metabolites of *C. purpureum*, the modes of action of these toxins were not known (Strunz *et al.* 1997). Many plant pathogenic fungi produce low mass toxins with varying activity and selectivity (Hallock *et al.* 1988, Manulis *et al.* 1984, Pedras *et al.* 1990, Venkatasubbaiah *et al.* 1992, Xiao *et al.* 1991), but specific differences in low mass phytotoxin production for the five tested isolates were not observed. It is possible that extraction of sterpuric acid and related compounds (eremophilanes) likely contributed to the observed low mass phytotoxicity since sesquiterpenoid compounds are known to be toxic to dicotyledonous plants (Strobel *et al.* 1992). If sterpuric acid was specifically extracted and detected by leaf disk assay, however, its presence was not confirmed in these studies. This finding suggests that potential biorational derivation for *C. purpureum* is unlikely based on the apparent lack of readily identifiable low mass phytotoxic activity detected by the utilized assays.

1.2 *Pectic enzymes as phytotoxins*

The relative activities of pectic enzymes were tested for each of the five *C. purpureum* isolates (2065, 2097, 2121, 2139, SL33) using a standardized induction medium containing citrus pectin as the sole source of carbon (Cruickshank 1987, 1990, Cruickshank and Wade 1980, Sweetingham *et al.* 1986). A standardized induction medium was required to ensure that pectic enzyme production did not vary with growth conditions, even though constitutive production of pectic enzymes by the fungus is a possibility (Cleveland and Cotty 1991, Miyairi *et al.* 1988, Neate *et al.* 1988). Subjecting crude culture filtrates to a leaf disk assay (Table 10) using the three indicator species (alder, maple and poplar) showed that each isolate expressed a reproducible level of phytotoxic activity. This activity decreased from isolate 2139 to 2097 to 2065 to SL33 to 2121. This is the same trend that was observed for the crude culture filtrates (Figure 4). Furthermore, the absence of indicator species selectivity in the pectic culture filtrate leaf disk assay supported that pectinases were effective phytotoxins at secreted concentrations for all tested species. This reinforced the presumably highly toxic nature of secreted pectinase (Miyairi *et al.* 1977, 1979, 1985).

The leaf disk assay results (Table 10) were supported by the electrolyte leakage data for the same set of crude filtrates (Table 11). The electrolyte leakage assay results, however, were not as well defined. It was apparent that isolates 2139 and 2097 did produce the highest level of measurable leaf damage, whereas the other isolates did not produce conductivity readings that were significantly different from the controls. This further detracted from the sensitivity of the electrolyte leakage assay, especially considering that known pectinase activity (as detected by the leaf disk assay) was detected for only two of the isolates. The pectinase standards (1 and 10 mg/ml) demonstrated that the concentrations of pectinase in the crude filtrate was less than 1 mg/ml. This must be carefully interpreted since endoPG production for other bacterial and fungal species has not been shown to cause disease symptoms at such small concentrations (Miyairi 1979, Pogson *et al.* 1991, Shanley *et al.* 1993).

To test the possibility that a fraction of the phytotoxicity was indeed due to pectin degrading enzymes a third assay was employed to measure pectinase activity in the cultures grown in the pectin induction medium. Preliminary activity of isolate culture filtrates were compared by measuring the decrease in the viscosity of a pectin substrate buffer over time (Table 12). If low mass toxins were the major contributors to the phytotoxic effects, they would have been detected by the leaf disk assay, but not by the viscosity assay because low mass toxins would not affect buffer viscosity. The viscosity assay trend (decreasing from 2139 to 2097 to 2065 to SL33 to 2121) was similar to the results for the leaf disk assay (Figure 5) suggesting that pectin degrading enzymes and leaf disk assay phytotoxicity were strongly associated, and that cell wall degrading enzymes were an important part of the phytotoxic process. Varying pectinase levels are common among many phytopathogenic fungi, however, this is the first report of differing toxic pectinase levels between isolates of *C. purpureum*, implicating a potential link of field virulence and pectic enzyme activity (Fernandez *et al.* 1993, Shivas *et al.* 1991, Wahlstrom *et al.* 1991, Wall *et al.* 1996).

It has been established that pectin degrading enzymes can play an important role in the pathogenicity fungi by degrading physical barriers which would otherwise prevent the fungus from growing intrusively into its host (Blanco *et al.* 1994, Miyairi *et al.* 1977,1979,1985, Miyairi 1988, Shamoun *et al.* 1993). Low mass toxins appeared to play less important of a role in the pathogenicity of *C. purpureum* as detected by these assays. It was considered likely that pectic enzyme production could be correlated to isolate virulence, as was observed in previous studies of *Aspergillus, Leptosphaeria* and *Sclerotium* virulence (Easton and Rossall 1985, Cleveland and Cotty 1991, Punja *et al.* 1985). If this were to hold true, then it may be possible to select local isolates of high virulence based on relative pectinase activity as lead isolates for development as mycoherbicides. This would also provide a way of assessing virulence for a local bioherbicidal post-inundation population. To fully test this hypothesis, twelve isolates were tested for pectinases and compared to tests of greenhouse virulence.

2.0 *Toxins and their roles in the pathogenicity of* Chondrosterum purpureum

From research reported by Miyairi and colleagues (1977, 1979, 1985), it has been hypothesized that endopolygalacturonase, an abundant form of pectinase produced by *C. purpureum*, is the major cell wall degrading enzymatic toxin produced by the fungus. The fungal disease process involves colonization of the stem and prevention of resprouting through necrosis of the meristematic tissues (Wall 1986, 1990, 1991, 1994). Infection of the vascular xylem permits translocation of endoPG, and other toxic metabolites, to remote areas of the tree (including the leaves), resulting in the characteristic silvering seen in some tree species. Since pectic carbohydrate polymers, such as the α–1,4-linked galacturonic acid structural sugar rhamnogalacturonan, are important in providing plant tissues with cellular adhesion, it is likely that degradation by pectinolytic enzymes such as endoPG is key to the pathogenic process, and contributes to the fungus' ability to penetrate cell walls. Gao and Shain (1994) observed decreased virulence with decreased endoPG production, yet disruption of the endoPG gene of the ascomycete *Cochliobolis carbonum* showed that the enzyme was not essential to pathogenicity when compared to the wild type (Scott-Craig *et al* 1990). For the latter example, it is possible that the monocotyledonous host plant tissues (maize, 8–9 % pectin) are not as easily degraded by endoPG as the pectin rich dicotyledonous plants (35 % by comparison, including weedy forest hardwood species).

As a preliminary assessment of the importance of pectin degrading enzymes to pathogenicity among *C. purpureum* isolates, pectin degrading activity was assayed for 12 isolates of the fungus collected from the southern Vancouver Island region. Dr. R. E. Wall, of the Pacific Forestry Centre, Victoria B.C., performed greenhouse pathogenicity assessments of these twelve isolates using red alder as the target species (unpublished data, Table 13). The percent mortality within twelve replicates of each isolate demonstrated that the tested isolates vary considerably in virulence (Wall *et al.* 1996, Shamoun *et al.* 1993, Ramsfield *et al.* 1996). The pathogenicity data were correlated to pectic enzyme toxin activity (as examined by the leaf disk, electrolyte leakage, and viscosity assays).

The leaf disk assay phytotoxicities (Table 14) share a trend similar to greenhouse mortality. Isolates 2140, 4u-88 and 2139, all with relatively high greenhouse virulence, showed a relatively high phytoxicity as detected by the leaf disk assay (Table 18). It was found that the majority of leaf disk assay phytotoxicity was due to the pectic enzyme fraction, since removal of low mass molecules from the filtrates resulted in no changes in isolate phytotoxicity trends. This finding supports a possible association between pectinase production and isolate phytotoxicity. Of note are the relatively high activities seen with isolates 2090 and 2123x. Although showing low mortality in the greenhouse assay, the culture filtrate phytotoxicities gave unexpectedly high values, though cause could not be identified.

The electrolyte leakage data of the same 12 isolates (Table 15) shows a trend similar to that of the leaf disk assay. There was a higher activity detected among the isolates of higher virulence in greenhouse pathogenicity tests, again supporting a possible link to phytotoxin production. The resulting conductivity increases for the more virulent isolates was likely due to the production of galacturonic acid residues from tissue pectin degradation. This may have resulted in greater osmolarity and conductivity for the isolates of higher pectinase production.

With a tentative link between isolate cell wall degrading pectic enzymes and phytotoxicity, a more rigorous correlation was made between greenhouse virulence and relative isolate viscosity activity (Table 16). Since this assay detects only pectin degrading ability (encompassing endo- and exopolygalacturonase, pectate lyase, and pectin esterase activities) as opposed to general phytotoxin activity, a more sound correlation between pectinase and virulence could be made. Using analysis of variance, relative pectinase activities (Table 16) were correlated to the initial rates of mortality (slope of mortality versus time, Table 19, Figure 6) for the 12 greenhouse isolates. The isolate mortality was associated with pectinase production (P-value of 0.0306, Table 19), thus it appears that a tentative link between pectinase production and isolate virulence had been established. The slopes of each isolates mortality, calculated using linear regression, were used as a representation of effective virulence for a given isolate at a time crucial to the disease process. Pectinase activity was also found to be correlated with the early seasonal mortality (July 1995, P-value = 0.0096, Table 19). This suggested that higher levels of pectinases could play an important role in disease establishment for the pathogen *C. purpureum*. Since endoPG is known to be the major form of pectinase produced by the fungus, this further indicates that the pathogenicity of *C. purpureum* may be linked to the cell wall degrading enzyme endopolygalacturonase (Miyairi 1979, 1985). Over all, there was a considerable range in greenhouse efficacy, yet there was a good correlation with pectinase phytotoxic activity.

3.0 *Summary and Conclusions*

Low mass toxins were detected within *C. purpureum* ethyl acetate culture fluid extracts. The lack of readily identifiable low mass phytotoxins, with the possible exception of sesquiterpenoids (e.g., sterpuric acid), suggested that the development of a new biorational from *C. purpureum* was unlikely, unless a highly active specific toxin could be identified, concentrated and produced by either synthetic or isolate over-production methods (Struntz *et al.* 1997). From comparison of pectinase activity and culture fluid toxicity, it appeared that phytotoxic effects were reproducible for a given isolate. This consistent ranking of phytotoxicity among isolates correlated better with pectinase activity than to low mass toxins.

Based on comparison of greenhouse virulence and viscosity assay results, pectinase production correlated well with isolate virulence (P-value = 0.0096). This could allow for prediction of *C. purpureum* bioherbicide isolate virulence for a given host species in a relatively short time (i.e., 2 weeks) when compared to conventional virulence assays (months in the greenhouse and/or field). This may provide a means for assessing the relative virulence of a local endemic populations of the fungus for use as future inundative bioherbicides.

Of the indicator species used in the assay, maple consistently was the most sensitive to toxin activity (Figure 1), although future testing of field isolates should be carried out on the intended host species. The media containing poplar host infusions enhanced the production of toxins (Figure 2). These observations may allow for prescription of specific isolates to be used as bioherbicides on a specific host weed species. This must be substantiated by field testing as the observed variation for a given leaf tissue could be due to structural/physiological characteristics inherent to that species rather than species selectivity.

Based on results of these and other studies, the major pectinase being responsible for phytotoxic activity is probably polygalacturonase activity. In conjunction with the leaf disk and viscosity assays, it was apparent that pectinase production played a much greater role in the culture filtrate phytotoxicity than did the low mass toxins (Figures 3 & 4). These findings are relevant to mode of action studies and enhancement of bioherbicidal efficacy. To rigorously test whether pectinase is the major genetically determined pathogenicity factor, strains which over-express or do not express pectinase must be constructed to assess the role of endoPG in pathogenicity.

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