A STUDY OF

Chondrostereum purpureum

AND ITS ROLE IN THE DECLINE OF WHITE BIRCH IN THUNDER BAY

by

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Abstract

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Key Words: birch dieback and decline, crystalline secondary metabolite, fruiting, pathogenicity, sexuality, silver leaf.

Various aspects of the biology of *Chondrostereum purpureum* (Pers.:Fr.) Pouz. and its role in dieback and decline of white birch (*Betula papyrifera* Marsh.) were studied. (1) Sixty-nine trees which exhibited symptoms of dieback and decline were sampled for *C. purpureum* infection in Thunder Bay, Ontario. Using a variety of sampling methods, including boring, and trunk and branch dissection, *C. purpureum* was isolated from 26 (38%) of 69 trees sampled. The increment borer sampling technique proved to be the most efficient method of isolating the pathogen, in terms of combining ease of execution and good success rate. (2) The pathogenicity of *C. purpureum* to birch seedlings was tested. All twenty-six seedlings infected with mycelium developed open wounds, 12 of which cankered, whereas the wounds in 26 controls closed within 4-6 weeks. Progress of the infection varied. Spread of the canker from the wound site (including stem girdling and dieback in two seedlings), spread through the xylem and formation of cankers further up the stem, and apparent containment of the infection at the wound site were observed. (3) The properties of white needle-like crystals which formed on heavy spore casts were investigated. The filamentous crystals were composed of a sesquiterpene compound ($C_{15}H_{26}O$, M^+ 222, m.p. 136 ^{o}C) similar to (+)-torreyol. (4) The sexuality of *C. purpureum* was investigated. The fungus is heterothallic, with tetrapolar sexual differentiation. Anomalies such as unilateral compatibility and unequal abundance of clamps and fruiting in compatible pairings were observed. (5) A method of inducing growth of *C. purpureum* basidiocarps and their storage was devised.

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Introduction

An air of mystery and contradiction surrounds the fungus *Chondrostereum purpureum* (Pers.:Fr.) Pouzar. Many aspects of its biology are unusual and puzzling. Although classified since the early 19th century in the genus *Stereum* (it has well-developed stereoid basidiocarps), it was later found to have an entirely different structure than true *Stereum* (Reid 1971) and Pouzar (1959) reclassified it into a new mono-typic genus, *Chondrostereum*. Its monomitic hyphal system (Reid 1971), single clamp connections (true *Stereum* species have multiple clamps) in both cultures and basidiocarps (true *Stereum* species have no clamps in basidiocarps) (Lentz 1971), the large saclike hyphal structures (i.e. vesicles) it produces in the sub-hymenial zone (Reid 1971), and its tetrapolar heterothallism (Robak 1936) all support its distinction from *Stereum*. Regarding its vesicles, all species which produce such vesicles are closely related; that is, except for *C. purpureum* (Stalpers 1978).

Taxonomy aside, its ecological role is also unusual. Well known for its apparent saprophytic role as a colonizer of freshly cut trees (Fritz 1954; Rayner 1977), it has long been recognized also as a wound parasite (Percival 1902). However, whereas most basidiomycete parasites are perthotrophs, i.e. they kill host material in advance of penetration, *C. purpureum* is a hemibiotroph, i.e. it

infects living tissue, eventually kills it, and then continues its development (including sporulation) on the dead tissue in competition with saprotrophs. This trophic behaviour is characteristic, not of Basidiomycetes, but rather of Ascomycetes (Luttrell 1974).

Consensus is lacking on the pathogenicity of *C. purpureum*. Its devastating impact on orchard trees (Setliff & Wade 1973) and hardwood stoolbed nurseries (Spiers 1985) has been reported, yet it was absent from a recent, extensive catalogue of diseases of trees and shrubs (Sinclair *et al*. 1987). Brooks and Bailey (1919) reported birch trees which had been rapidly killed by mycotoxins released by *C. purpureum*. Wall's (1986, 1991) inoculation tests with yellow birch (*Betula alleghaniensis* Britton) resulted in cankers (some which healed) but no mortality.

This study was undertaken with the aim of exploring a number of aspects of the biology of *C. purpureum*. Chapter 1 reports on *C. purpureum*'s association with decline of white birch (*Betula papyrifera* Marsh.). The subject of Chapter 2 is a pathogenicity test with young birch. Chapter 3 describes an investigation of a crystalline compound produced by germinating basidiospores of *C. purpureum*, and Chapter 4 explores the sexuality of *C. purpureum*. In light of the need to conduct further studies on *C. purpureum*, Chapter 5 presents methods of growing and preserving basidiocarps.

A Survey of *Chondrostereum purpureum* Associated With Birch Decline

Chondrostereum purpureum was first recognized in 1902 as the wound pathogen which caused silver leaf disease of fruit trees such as plum and peach (Percival 1902). Later, the fungus was reported worldwide on a wide range of woody plants of the temperate zone, including poplar, plane, cherry, birch, horse chestnut, gooseberry (Brooks & Bailey 1919), balsam fir (Smerlis 1961), maple, mountain ash (Pilley & Trieselmann 1969), and apple (Setliff & Wade 1973). In New Zealand *C. purpureum* is the principal invader of wounds in eucalypts (Gadgil & Bawden 1981), and silver leaf is the most serious disease affecting pole production from poplar and willow stoolbed nurseries (Spiers 1985). The first North American report of silver leaf disease was by Güssow (1911) who found it on an apple tree in Nova Scotia.

Although it has a very wide host range, study of the pathogenicity of *C. purpureum* to species other than fruit trees has been limited (e.g. Minervini & Bisiach 1980 - cankers in a *Platanus* sp.; Wall 1986 - cankers in yellow birch). The fungus has been tested as a silvicide on *Prunus serotina* Ehrh. (de Jong *et al.* 1990a, 1990b) and other hardwoods (Wall 1990).

As the name suggests, silver leaf disease results in a silvery appearance of the leaves of the infected tree. The silvering results from the action of a protein (an endo-polygalacturonase) which disrupts the middle lamella

between the epidermal cells and palisade cells, causing the two layers to separate. This change in cell arrangement results in changed reflectivity of the leaf surface; thus the silvery appearance. The endo-PG also has a macerating action in several plant tissues (Miyairi *et al.* 1977). Brooks and Brenchley (1929) found evidence of an additional toxin which killed leaf and flower tissue in Victoria plum. Other common symptoms of silver leaf disease include branch dieback, overall tree decline, and cankers. Internally, the wood invaded by the pathogen often becomes darkly discoloured by gums and resins (Brooks 1913).

Dieback and decline of white birch is common throughout both the natural forests and urban areas of northern Ontario. The best-documented episodes of widespread dieback and decline of white birch in Canada occurred in the period from the early 1930's to the late 1950's. Symptoms included thinning foliage, curled or chlorotic leaves, dieback of branches in the upper crown, and the production of clumps of foliage in the lower crown. No single causal agent was determined, in spite of years of intensive research (Houston 1987).

White birch is an important shade tree in Thunder Bay, Ontario. Many streets are lined with birch which were planted by the City beginning in 1933. Dieback and decline of all age classes of these trees was noted throughout the city (McLaughlin & Setliff 1990).

In September, 1986, *C. purpureum* was observed fruiting on a sickly young white birch on the Lakehead University campus. In the late summer of 1988, two more trees were observed with *C. purpureum* basidiocarps. One of these trees was 55 years old and had died during the summer of 1988. The other was a much younger tree that was dead in the upper crown.

During the summers of 1989 and 1990, surveys to assess this fungus' association with white birch trees that exhibited symptoms of decline and dieback, and with recently killed trees, were conducted in Thunder Bay.

Materials and methods

Tree Selection. The primary criterion applied for the selection of trees for investigation was crown dieback. Trees with trunk wounds and decline symptoms (e.g. trees with small foliage and/or thin crowns) were also eligible for selection (Fig. 1.1). The silver leaf symptom fails to occur in birch, even when heavily infected with the pathogen (Brooks & Bailey 1919; Brooks & Storey 1923).

Sampling Techniques. Three survey methods for isolating the fungus in symptomatic trees were employed: increment borer sampling, trunk dissection, and dissection of newly killed branches. The objective in increment borer sampling was to extract a core sample from columns of discoloured wood associated with wounds (if present, the fungus will be

isolated from the brown-coloured wood (Brooks 1913; Schlechte 1981)). A small area of outer bark was cut from a spot adjacent to a wound or branch stub on selected trees. An increment borer rinsed with 70% ethanol was used to extract the wood cores. The cores immediately were transferred to sterilized test tubes for transport to the lab. Normally, only one core was extracted from each tree, however, if the first attempt failed to yield discoloured wood, a maximum of two additional samples was taken. Both discoloured and clear-wood cores were tranferred to malt extract agar (MEA) plates. This method was employed during the 1989 field season.

Several of the trees which had been sampled by increment borer during the summer of 1989 were dead or very near death by July of 1990. Seven of these previously sampled trees were dissected. In addition, four previously unexamined symptomatic trees were dissected. The trees were felled, sawn into manageable lengths and then cut longitudinally with a bandsaw. Isolation attempts were made from exposed discoloured wood.

The third survey technique, dissection of newly killed branches, was employed in August and September of 1990. Branches which had leafed out in the spring but had died during the summer were conspicuous because of the premature browning of the leaves (flagging). Thirteen of these

branches were dissected and examined in the same manner as described above for trunks.

Isolation Technique. The wood cores extracted by increment borer were placed in petri dishes containing 1.25% (MEA) plus 25 ppm benomyl and 250 ppm guaiacol. The fungicide benomyl was added in order to inhibit the growth of moulds which might have otherwise prevented the growth of *C. purpureum* or masked its appearance. Benomyl acts to slow the growth on a wide range of fungi but has a reduced effect on Basidiomycetes (Kendrick 1985). Guaiacol was added to the media as an indicator because it turns orange-brown in the presence of *C. purpureum* (Bishop 1979). The dishes were incubated on a laboratory bench at ambient room conditions.

As fungal growth from the wood core was observed, isolations were transferred onto 1.25% MEA in petri dishes and left on the laboratory bench to grow until they reached a stage of development when they could be identified. Isolations were recorded as originating from either clear wood or discoloured wood.

Benomyl and guaiacol were not added to the media used in isolation attempts from the trunk and branch sections. Slivers of wood were aseptically cut from discoloured wood and placed onto 1.25% MEA in plates and left on the lab bench. Macroscopic identification of the pathogen was made as the fungus grew out of the wood slivers.

Figure 1.1. A symptomatic tree among apparently healthy ones on the Lakehead University campus.

Results and discussion

Sampling Techniques. Successful isolation attempts were made from material collected by all three sampling techniques. The increment borer technique was not as reliable a method for locating discoloured wood as were the dissection techniques; discoloured wood was located in only 39 of 52 trees sampled (Table 1.1). This quick, non-destructive technique proved, however, to be the most efficient method of isolating the pathogen in terms of ease of execution and success rate. Wood cores from 17 (33%) of the 52 trees sampled yielded *C. purpureum* in culture. The

17 positive isolations represent a 44% isolation success rate from the 39 discoloured wood cores extracted by this method.

Trunk dissection was a reliable method of locating possible infection sites but also was labour-intensive and by definition a destructive sampling technique. The pathogen was isolated from 36% of the trees sampled by this method. Branch dissection was as effective a technique as trunk dissection for locating possible infections. Also, it was a less destructive technique but had the disadvantage of limited access to dead branches high in the crown. The success rate for isolating the pathogen from trees sampled by this method was 23%.

Table 1.1. *Chondrostereum purpureum* isolations from symptomatic white birch.

Sampling Technique	# of trees sampled	Success in locating discoloured wood	# of positive isolations
Increment Borer	52	39	17
Trunk Dissection	11[a]	11	4[b]
Branch Dissection	13	13	3

[a] Seven of these trees were among those previously sampled by increment borer.

[b] Two of the positive isolations came from trees which had been sampled by increment borer but failed to yield *C. purpureum* by that method. In fact, discoloured wood had not even been located from one of these two. The fungus was isolated from one of the four previously unexamined trees that were dissected.

The trunk and branch dissection techniques allowed for determining the age of the wounds which had served as entry points for the pathogen. Wound ages ranged from a 1-year-old wound on a dying branch (Fig. 1.2) to an 11-year-old trunk wound tree with advanced dieback.

Isolation Technique. *Chondrostereum purpureum* was isolated from discoloured wood associated with both branch and trunk wounds. In no case was the fungus isolated from wood that was free of discolouration. Most success came from isolation attempts made at the edge of the discoloured portion of the wood.

In the earliest isolation attempts, the guaiacol reaction, suppression of undesired moulds by benomyl, and fruiting in culture were aids to identification. However, it became evident that in most cases the rapid growth rate and distinctive appearance of *C. purpureum* cultures provided sufficient evidence by which to identify the fungus. The advancing hyphae were distant, the margin was uneven, and in many cases there was a slight clock-wise rotation to the direction of hyphal growth at the leading edge of the colony. This last feature was most noticeable when the mycelia had grown out 2-3 cm (Fig. 1.3).

Chondrostereum purpureum grew quickly, even in the presence of 25 ppm of benomyl and usually emerged within 2-3 days of plating. The growth rate on MEA was always

Figure 1.2. Stages of limb dieback caused by *C. purpureum*: death of the infected branch and dying foliage and twigs on the distal portions of the limb.

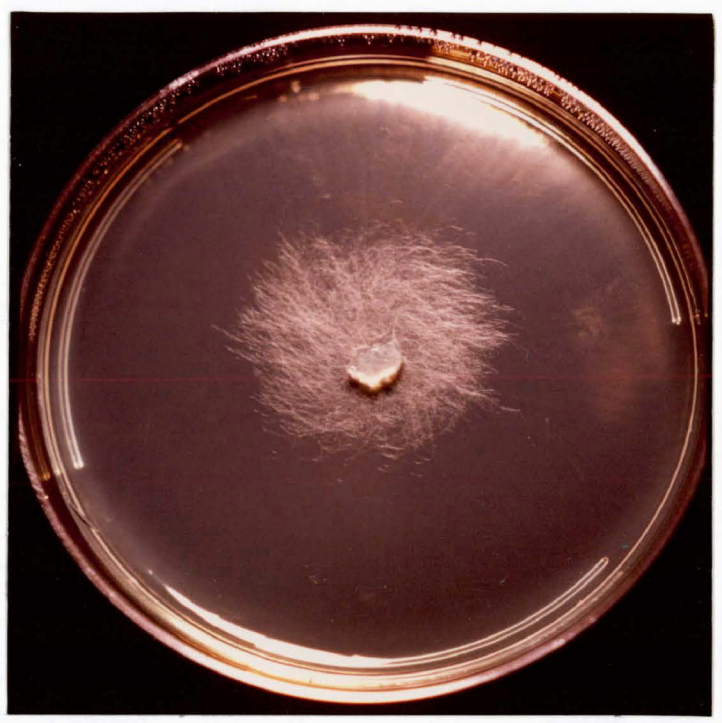


Figure 1.3. Distinctive cultural appearance of *C.purpureum* with uneven margin and clock-wise rotation of leading hyphae.

rapid. When isolated to the centre of a 8.5 cm petri dish, the culture covered the medium surface within 4-5 days at 22°C.

In cases which required further verification, the conclusive characteristic was fruiting. Basidiocarps that formed in culture were atypical of those found in nature. After about 3 weeks of growth in the petri dishes, small cream-coloured mounds formed along the outer edges of the agar and on mycelia growing up the sides of the dish (Fig. 1.4). Microscopic examination revealed vesicles, basidia, and basidiospores characteristic of *C. purpureum*.

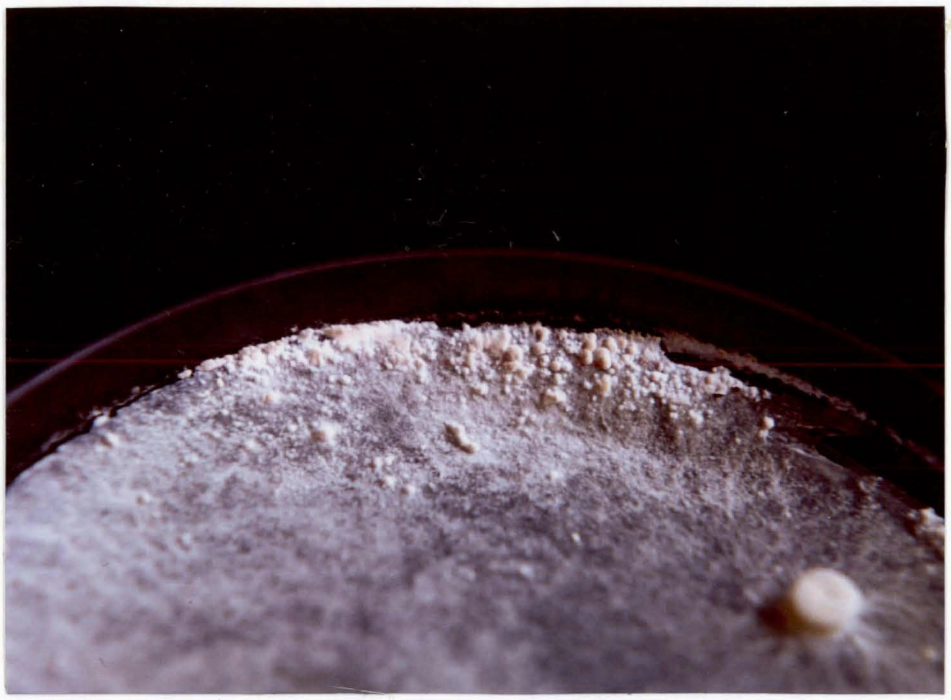


Figure 1.4. Typical *C. purpureum* basidiocarp as it forms in culture.

Association with Disease. This study is not the first to identify an association between *C. purpureum* and defects in white birch. In cull studies conducted by Basham and Morawski (1964), *C. purpureum* ranked fourth (13 of 107 identified infections) in basidiomycetes associated with various types of trunk defects. *Chondrostereum purpureum* ranked second (four of 22 identified infections) for the defect incipient yellow rot, and first (seven of 24 identified infections) for the defect brown stain.

Given this clear association, it seems puzzling that *C. purpureum* was not isolated from dead branches sampled in earlier investigations of dieback (Horner 1953). This may

be because the toxins produced by the fungus are transported in the xylem from the site of infection to the distal portions of the crown or branch (Cunningham 1922) and can, therefore, kill from a distance. Branches killed in this way will bear no signs of the pathogen, but isolation attempts may yield a variety of non-pathogenic saprophytes, as was the case in earlier investigations (Hansbrough 1953; Horner 1953).

In this study, trees infected in the trunk exhibited a general decline, characterized by a sparse crown with small leaves and dieback either from the top or the bottom of the crown but not aleays limited to only one portion or side. Trees with branch infections had flagging or dieback of the infected branches but the rest of the crown often appeared healthy. This pattern of decline and dieback symptoms in diseased trees was very close to that observed by Cunningham (1922) in New Zealand (except for the silver-leaf symptoms), and to that observed in a newly planted apple orchard in Wisconsin (Setliff & Wade 1973).

Bronze birch borer (*Agrilus anxius* (Gory)) galleries were often found in the infected trees. Borer damage is a conspicuous feature commonly observed in dying birch. So noticeable is this association that the borer was believed to be the primary agent causing birch dieback and mortality (Hawboldt 1947). Although the borer may be a major factor in the death of trees already affected by dieback, it is

not the primary agent which initiates the condition (Hawboldt & Skolko 1947; Barter 1953; Quirke 1953).

Many questions regarding the role of *C. purpureum* in birch dieback and decline remain unanswered. Although the results of this study demonstrate association between *C. purpureum* and birch dieback and decline, the pathogenicity of the fungus to healthy trees has not been explored adequately. Wall's (1986, 1991) pathogenicity test with young yellow birch resulted in cankers but no mortality after 15 months. Early results of a pathogenicity test currently being conducted with white birch seedlings (see Chapter 2) have demonstrated *C. purpureum*'s ability to cause cankers, dieback, and tree mortality.

Evidence and observations gathered in this survey have generated a number of hypotheses regarding the pathogenic role of *C. purpureum* in white birch. One hypothesis is that *C. purpureum* is a fast-acting, virulent primary pathogen which produces mycotoxins capable of quickly killing a branch or entire tree (depending on the site of infection) by itself (e.g. Fig. 1.2). Another hypothesis is that the fungus may predispose the tree to attack by other organisms (e.g. bronze birch borer) which, together with the systemic action of the mycotoxins, result in a steady decline (Fig. 1.5).

Figure 1.5. Decline symptoms throughout a tree infected with *C. purpureum*.

Tree mortality factors may also include: (1) the tree's effectiveness in compartmentalizing the fungus; (2) seepage of mycotoxins from the compartmentalized zone into the sap flow; (3) multiple infections over several seasons resulting in a mycotoxin-tolerance threshold being exceeded; (4) loss of water-transporting tissue to compartmentalization; (5) and other contributing agents (e.g. weather, trauma) which reduce tree vigour and thereby allow the rate of spread of the fungus in the tree to exceed the tree's ability to contain it by compartmentalization.

A Study of the Pathogenicity of *Chondrostereum purpureum* to White Birch

The association of *Chondrostereum purpureum* with dieback and decline of white birch has been reported by McLaughlin and Setliff (1990) and Setliff and McLaughlin (1991). Although observation of a persistent fungal association with a specific disease is not proof of pathogenicity, it is an important first step in determining a causal relationship.

Some investigations into the pathogenicity of *C. purpureum* to other tree species have been carried out. Brooks and Bailey (1919) proved the pathogenicity of *C. purpureum* to the plum varieties Victoria and Czar in an application of Koch's postulates. Likewise, Wall's (1986, 1991) experiments with yellow birch and beech (*Fagus grandifolia* Ehrh.) showed that *C. purpureum* was capable of producing stem cankers. de Jong *et al.* (1990a) demonstrated the pathogenicity of *C. purpureum* to black cherry in biocontrol trials in the Netherlands.

Dieback and decline of birch in northeastern North America has been a recognized problem since the 1920's. Attempts to provide a satisfactory explanation of the cause of this phenomenon have so far been unsuccessful, in spite of intensive investigations into the abiotic and biotic aspects of the problem (Houston 1987).

Given the proven association of *C. purpureum* with birch dieback and decline, and the fact that *C. purpureum* has never been investigated as a possible cause of this

problem, it is important to determine the virulence of this pathogen to birch. The objective of this study was to apply Koch's postulates and determine if *C. purpureum* was capable of causing the symptoms observed in white birch in the field, as reported in this study and in Chapter 1.

Materials and methods

Experimental design. The experiment was executed in two stages using a completely randomized design. The treatment structure was factorial (2 X 2) in the second stage.

Stage 1. Stage 1 comprises two growing seasons and the treatments applied during this period. Birch seedlings were grown in the greenhouse for six months, by which time they ranged in height from 15 to 32 cm tall. They were then put in a growth chamber under short days (6 hours) and low temperature ($4^{\circ}C$) to induce dormancy. After 3.5 months they were transferred to the greenhouse to break dormancy. The treatments were applied 2.5 months into the second growing season, after shoot elongation had ceased. Two treatments were assigned randomly to 52 seedlings, which at this time ranged in height from 44 to 68 cm tall. Twenty-six of the seedlings were inoculated with *C. purpureum* mycelium grown on sterile malt agar, and a control treatment of sterile malt agar was applied to the other 26.

Inoculation technique. Mycelium from *C. purpureum* (isolate JAM-3) isolated from a dying birch (described

later) was used as inoculum. The seedlings, ranging in height from 44 cm to 68 cm, were inoculated approximately 2-5 cm above the root collar. The bark was first cleaned with 70% ethanol and then a 1 mm X 5-7 mm axial cut was made through the bark in the stem, just deep enough to penetrate slightly into the xylem tissue. Plugs of *C. purpureum* mycelium grown on 1.25% malt agar were cut with a 2-mm cork borer. The inoculum was applied to the wound and then the wound was covered with Parafilm (American Can Company) to maintain moist conditions and to prevent contamination from air-borne moulds or other fungi. The control treatment differed only in that plugs of sterile malt agar without mycelium were applied to the wounds; otherwise the seedlings were treated identically.

The seedlings were kept in the greenhouse for two months after inoculation. They were then transferred to a growth chamber under short days (6 hours) and low temperature ($4^{o}C$) conditions to induce dormancy.

Stage 2. After three months, the seedlings were returned to the greenhouse and put under 16 hour day light conditions. When the seedlings had flushed and the initial leaves were expanded, a second experimental treatment was applied (Table 2.1). Thirteen each of the inoculated and the non-inoculated seedlings were put under water stress. They received water only when more than 50% of the leaves

were wilting. The other 26 seedlings were watered every 4–5 days, or as necessary to prevent water stress.

Table 2.1. Allocation of treatments in Stage 2.

Treatment Combination	Inoculated	Moisture Stress	Number of Seedlings
TC1	yes	no	13
TC2	yes	yes	13
TC3	no	no	13
TC4	no	yes	13

Response variables. In the latter part of Stage 1, the seedlings were observed to see if the wounds had closed (i.e. calloused over), or conversely, they had remained open or a canker had developed. In Stage 2, wound closure and canker development were monitored. In addition, height increment was compared by the analysis of variance (ANOVA).

Representative inoculated and non-inoculated seedlings were dissected and the extent of wood discolouration in each was examined. The spread of the infection through the bark was also examined. Attempts to isolate *C. purpureum* from cankers and discoloured wood were made, in conformity with Koch's postulates.

Results

Field observations. In July, 1989 a dying white birch (approx. 25 years old) on the Lakehead University campus was dissected. The crown, where it was still alive, bore very few leaves (Fig. 2.1). *Chondrostereum purpureum*

basidiocarps were growing near a branch stub on a lower limb. Removal of outer bark from the limb and main stem revealed a large area of necrotic bark which extended almost 1 m down the branch and stem (Fig. 2.2). Internally, a column of discoloured wood extended downward approximately 125 cm from the infected branch (Fig. 2.3). *Chondrostereum purpureum* was isolated from the necrotic bark and all areas of the column of discoloured wood.

Wounds and cankers. The difference in wound closure between inoculated and non-inoculated seedlings was distinct (Table 2.2). None of the wounds in the inoculated seedlings (i.e. TC1, TC2) closed and some developed into cankers (Fig. 2.4). All of the wounds in the non-inoculated seedlings (i.e. TC3, TC4) closed within 4-6 weeks of the treatment (Fig. 2.5).

Table 2.2. Wound closure

Treatment Combination	Closed Wounds	Open Wounds	Cankers
TC1, TC2	0	26	12
TC3, TC4	26	0	0

Progress of infection. Cankers developed in 12 of the 26 inoculated trees. Some cankers grew only slightly and were soon contained by callous but others continued to enlarge during both Stage 1 and Stage 2 (Fig. 2.6). Some which had spread during Stage 1 appeared to have been contained during Stage 2 (Fig. 2.7).

Figure 2.1. Dying tree infected with *C. purpureum*

Figure 2.2. Canker spreading down from infected branch.

Figure 2.3. Column of discoloured wood extending from branch wound.

Figure 2.4. Canker spreading after inoculation.

Figure 2.5. Wound closure in non-inoculated seedling.

Figure 2.6. Canker continuing to spread during Stage 2.

Figure 2.7. Spread of canker stopped during Stage 2.

Infection spread through both the wood and the bark. In three cases, infections which appeared to be contained at the wound site reemerged at another point on the stem, forming cankers or girdling small branches (Fig. 2.8). Two of the three were seedlings which were receiving water regularly; the third was under water stress. In the other nine cases, the infection spread through the bark from the wound site.

One tree died shortly after it was returned to the greenhouse. The buds began to open but before the leaves had emerged the fungus girdled the stem and killed the upper portion of the seedling. Epicormic buds emerged below the infection point but they died by the time the leaves had enlarged to approximately 1 cm long. Another tree died back above the wound but was successful in sending out long shoots from below the wound. Although healthy at first, these too began to wilt and die back (Fig. 2.9).

Dissection of infected trees revealed that the infection had spread longitudinally within the xylem through the vessels (Fig. 2.10) and also centripetally, invading xylem distal to the wound site (Fig. 2.11). *Chondrostereum purpureum* was isolated from discoloured wood in the stem and roots as well as in the dead bark of the cankers.

Figure 2.8. Canker formation further up stem.

Figure 2.9. Dieback of an infected seedling.

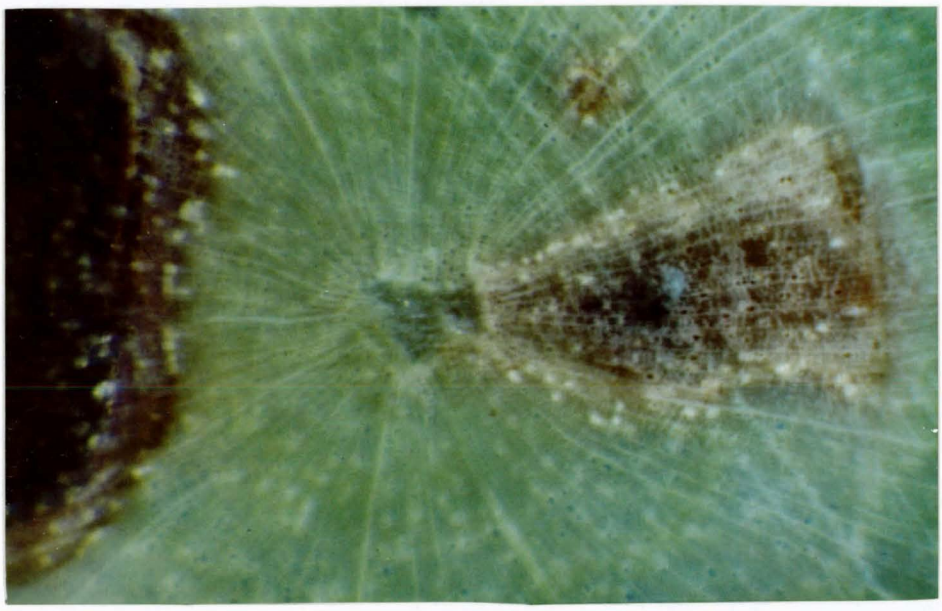


Figure 2.10. Vessels plugged by mycelium. x 25

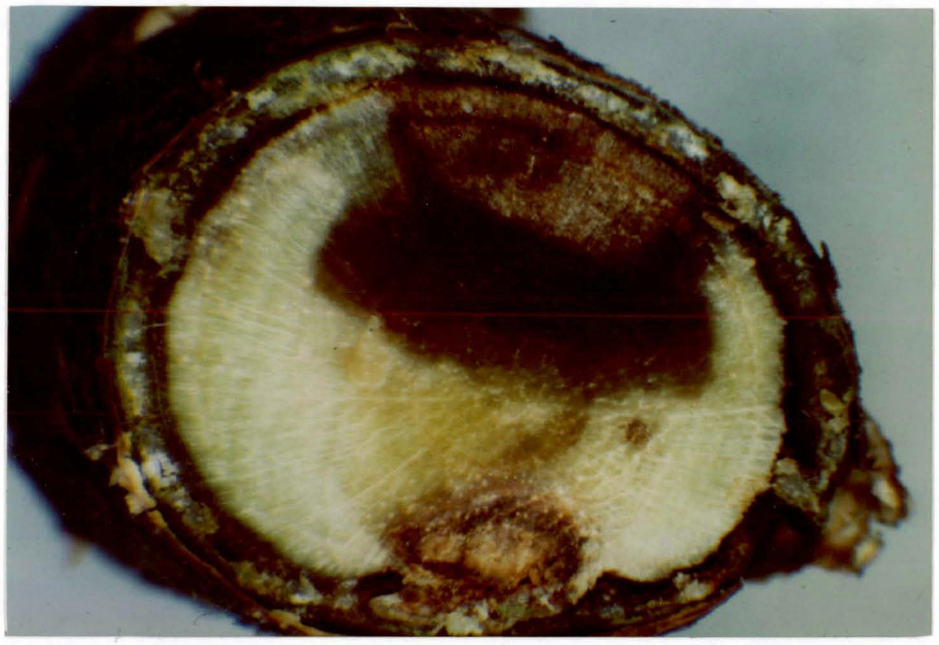


Figure 2.11. Spread of the infection from the wound to the other side of the stem and the resultant extensive invasion of xylem. x 13

Foliar symptoms. Leaves on seedlings in an advanced stage of disease (Fig. 2.12) differed in colour and sheen from those on healthy seedlings (Fig. 2.13). The leaves of the diseased seedlings were a darker shade of green but unevenly pigmented (i.e. lighter-coloured blotches between the veins). They were also duller, lacking the sheen of healthy leaves. In addition, while subjected to water stress, the foliage of healthy trees wilted more readily than foliage of infected trees (Table 2.3; Appendix I).

Figure 2.12. Leaf of seedling in advanced stage of disease.

Figure 2.13. Healthy leaf of a non-inoculated seedling.

Table 2.3. Water demands of inoculated and non-inoculated seedlings subjected to water stress over a period of 42 days.

Treatment Combination	Mean number of wilts	Mean number of days between watering
TC2 inoculated	3.23**	11.85*
TC4 non-inoculated	4.54	8.75

*significant difference ($\alpha < 0.01$)
**significant difference ($\alpha < 0.001$)

Height increment. The height increment measurements (Table 2.4) represent average shoot growth during the first 6 weeks of the first growing season following inoculation. The seedlings ranged in height from 51.2 to 116.5 cm when height increment was measured. Analysis of variance (Appendix II) of the height increments revealed that only water stress had influenced growth significantly. No effect of the pathogen or interaction of the pathogen with water stress was detected at this early stage of the study.

Table 2.4. Growth response of white birch seedlings to *Chondrostereum purpureum* inoculation and water stress.

Treatment combination	Average height increment since inoculation
TC1 - inoculated + water	30.4^{a}
TC2 - inoculated + stress	15.4^{b}
TC3 - non-inoculated + water	32.6^{a}
TC4 - non-inoculated + stress	18.8^{b}

$^{a\ b}$ significant difference between values with different superscript.

Discussion

Inoculation. In tests of pathogenicity, the method of inoculation is sometimes questioned. Even a saprophytic nonpathogen can produce wound expansion if a large mass of mycelium is introduced into the wound (Manion 1981). In nature, infection with *C. purpureum* is caused by spores, not mycelium. Most infections probably result when more than one spore infects a wound; isolates invariably fruit in culture, indicating dikaryotic mycelium. The literature does not contain data regarding the minimum spore load required to result in infection.

An attempt to inoculate the seedlings with a spore suspension during the second growing season (prior to inoculation with mycelium) failed, probably because the concentration of spores was too high (2.25×10^6 per ml). Stanislawek *et al.* (1987) also found spore inoculum unreliable in tests with *C. purpureum*. Grosclaude *et al.* (1973) concluded that poor infection success was the result of too many spores in the suspension. An inhibitory effect was apparent when spores were cast in heavy deposits on malt agar.

The 2 mm inoculum plugs used in this test were adequate to infect successfully the small wounds in the seedlings. They were not so massive, however, as to overpower initially the defence response. No trees died until the growing season following inoculation and dissection of the

wound area revealed that gum barriers had formed around the wound, initially resisting spread of the infection.

Wound closure and progress of infection. Although none of the inoculated wounds closed during the experiment, the progress of callous development in some, even those with small cankered areas, indicated that closure was not only possible but likely. However, as evidenced by reemergence of the cankers at points distant from the wound, callous formation around the open wound was not an indication that the fungus had been successfully compartmentalized (Shigo 1984) within the stem.

According to Shigo's (1984) compartmentalization theory, trees react to injury or infection by producing gum barriers which isolate the injured or infected tissue from the rest of the tree. This compartmentalization can be modelled as composed of four walls: Wall 1, which resists vertical spread; Wall 2, which resists inward spread; Wall 3, which resists lateral spread; and Wall 4, the strong "barrier zone" which is produced by newly formed cells in the cambium and which resists spread into tissue produced after the injury or infection.

Dissection of seedlings which exhibited reemergence of cankers further up the stem revealed that the fungus had overcome Wall 2, and the infection had spread to the other side of the stem (Fig. 2.11). This invasion met little resistance and quickly spread up the stem, probably

outstripping the seedlings ability to produce a Wall 1 reaction zone adequate to retard the spread of this aggressive fungus.

The most interesting point is that the fungus penetrated an apparently well-formed Wall 2 reaction zone and massively invaded the wood on the other side of the stem before the tree could react to contain it, especially with the radial reaction zones (i.e. Wall 3). In addition, the reemergence of the canker necessitated penetration of the barrier zone, Wall 4, normally the strongest barrier. Further, two of the three seedlings in which a canker reemerged up the stem were of those which had been watered regularly and had produced above average height increments. This is evidence that *C. purpureum* is an aggressive pathogen capable of causing disease in healthy plants, and not simply a nonaggressive pathogen that can only attack plants weakened or predisposed by stress (Schoeneweiss 1981).

Some seedlings that failed to exhibit reemergence of cankers had infection columns on the other side of the stem but their lateral spread had been resisted. Genetic variation among the individual seedlings may play a role in resistance to the fungus.

Chondrostereum purpureum can live for many years in a tree. Wall (1991) isolated *C. purpureum* 6 years after inoculation. In the course of conducting the survey

reported in Chapter 1, *C. purpureum* was isolated from a column of discoloured wood associated with an 11-year-old branch stub. Dissection of dying trees sometimes revealed a succession of barrier zones, indicating alternate compartmentalization and outbreak, with the fungus gradually invading more and more of the stem. This may explain the gradual decline and then sudden death of infected trees.

Foliar symptoms. Neither leaf silvering nor other foliar symptoms have been reported in the *C. purpureum*-infected birch species studied by Brooks and Storey (1923) and by Wall (1986, 1991). Silvering was not observed in this study of white birch, nor in the urban survey reported in Chapter 1, but heavily infected trees did exhibit foliar symptoms in the late stages of the disease. The leaves of the diseased seedlings were smaller than normal, dark green and unevenly pigmented as well as duller, lacking the sheen of healthy leaves. These symptoms were also observed in the mature, diseased street trees.

The causes for these symptoms in white birch remain to be explored. The absence of silvering, however, does not imply absence of effect from the infection. Spiers *et al.* (1987) found that *Populus euramericana* (Dode) Guinier, which silvered only slightly and only in the late stages of the disease, produced leaves 30% smaller than those of uninfected trees. They also related the severity of foliar

symptoms to the extent to which the wood had been invaded. This observation agrees with those of Bennett (1962), Schlechte (1981), and with observations made in the current studies of *C. purpureum* infection of white birch.

Whether the foliar symptoms were the direct result of toxins released by the fungus is unknown. Disruption of the water conducting capacity of the xylem due to physical occlusion of vessels by mycelium can result in water stress, which in turn can cause stomatal closure, decreased photosynthesis and respiration, and growth inhibition. Ultimately, cell death can result from the metabolic changes induced by water stress (Levitt 1980).

Current knowledge does not permit discrimination between the changes caused by water stress from those caused by mycotoxins (Spiers *et al.* 1987). It is noteworthy, however, that the non-inoculated seedlings subjected to water stress (TC4) did not exhibit these symptoms, even though they reached wilting point more often than the infected seedlings during the duration of the experiment.

Spiers *et al.* (1987) found that the transpiration rate of infected trees was lower than that of healthy trees; that is, the stomata of infected trees tended to remain closed either because of water stress or the action of mycotoxins. Brooks and Storey (1923) found the opposite; silvered foliage wilted more readily than healthy foliage. The findings of the current study (i.e. that the foliage of

infected trees wilted less readily than healthy foliage) are opposite to those of Brooks and Storey (1923) and are in agreement with those of Spiers *et al.* (1987).

Potassium intake by the guard cells is a key factor in stomatal opening. Active transport of the electrolyte K^+ into these cells causes increased turgor pressure and stomatal opening (Larcher 1980; Villee *et al.* 1985). Some mycotoxins can cause rapid loss of electrolytes, especially K^+, from plant cells (Gardner *et al.* 1974). Spiers *et al.* (1987) reported that leaf cell death seemed to be due to loss of electrolyte, evidenced by increased electron density of the cytoplasm and reduced cytoplasmic volume.

Endopolygalacturonase I (EC 3.2.1.15) (Miyairi *et al.* 1985), the toxin responsible for leaf silvering is the only toxin released by *C. purpureum* that has been investigated, although as early as 1929 the presence of at least one other, apparently heat-resistant toxin, was known (Brooks & Brenchley 1929). The possible effects of other toxins may have been overlooked because the attention of researchers has been drawn to the more dramatic silvering symptom. Some toxins with only slight initial effect are known to accumulate in plant tissue, resulting ultimately in cell death and systemic toxaemia (Scheffer & Livingston 1984).

Exactly what role the toxins of *C. purpureum* play in producing the symptoms observed in the birch seedlings remains unknown. However, the results of this experiment

suggest that they do contribute, possibly in causing stomatal closure which can lead to reduced photosynthesis, depletion of food reserves and starvation (Levitt 1980).

Height increment. Water stress had a highly significant effect on height increment. Water is essential to the photosynthetic process, affecting both the primary metabolic processes of the cell and the activities of enzymes and other biochemical events in the secondary reactions are affected by water availability. In addition, stomatal closure resulting from loss of cell turgor and the resultant interruption of the CO_2 supply restrict the photosynthetic process (Larcher 1980).

Six weeks into the growing season following inoculation of the seedlings with *C. purpureum*, no significant difference between inoculated and non-inoculated was evident. This experiment has not been terminated, rather it is being carried on and the seedlings will be observed for differences in growth rate. In light of the factors discussed above (i.e. occlusion of vessels by mycelium, effects of toxins on stomatal closure), it is probable that, given time, treatment effects attributable to *C. purpureum* infection will be observed.

An Investigation of Crystals Produced by *Chondrostereum purpureum*

Fungi are "famous" for producing many compounds of unknown function (Griffin 1981). Many of these compounds are secondary metabolites derived from primary metabolism precursors and include phenolic compounds, isoprenoids (e.g. terpenes), and antibiotics. They are produced when excess carbon is available after growth has ceased due to the limitation of some other factor (e.g. nitrogen) (Griffin 1981). In most cases, the specific role of the compound in the functioning of the cell is unknown (Smith & Berry 1974), which may be the main basis for their classification as secondary (Griffin 1981).

Many fungi, when grown in culture, produce secondary metabolites that form crystals in agar medium or on aerial hyphae. The crystals may be needle-like, cubic, or plate-like (Stalpers 1978). Stalpers sometimes found an unspecified type of crystal in aerial hyphae of cultures of *Chondrostereum purpureum* in his extensive study of wood-inhabiting fungi in pure culture.

In the course of studies on *C. purpureum* I observed white, needle-like crystals projecting from the surface of a plate of malt agar (MEA) medium which had been inoculated four weeks earlier with a suspension of basidiospores. The suspension held approximately 2.25×10^6 spores per ml and was being tested for spore viability as part of a pathogenicity test. The plate had been incubated on a lab bench at room temperature. Later, the crystals were again

observed on heavy spore casts from the same fungus on 1.25% MEA after three weeks incubation in a growth chamber under constant light (GE Chroma 50 fluorescent lamps) at 18°C.

Brooks and Brenchley (1929) demonstrated that *C. purpureum* mycelium releases at least two mycotoxins, one a pectinase (endopolygalacturonase) responsible for leaf-silvering (Miyairi *et al* 1977), and one which induced discolouration in plum leaves. The exact identity and origin of these chemicals, in particular the second, has yet to be determined. More recently, a unique group of sesquiterpene secondary metabolites of this fungus was discovered (Ayer *et al*. 1981).

The discovery of the crystals raised the question of whether they were a crystalline form of one of the phytotoxic compounds or sterpurenes produced by *C. purpureum*. This study was undertaken to investigate the growth and morphology of the crystals and to determine their composition and biochemical origin.

Materials and methods

Crystal production. *Chondrostereum purpureum* basidiocarps from thirteen sources (12 from white birch and one from trembling aspen (*Populus tremuloides* Michx.)) were used in this study. The basidiocarps were soaked in water until they were swollen (15 minutes was adequate) and then attached with petroleum jelly to the underside of petri

dish lids. The plates were placed in a growth chamber under constant light and at a temperature of 18°C. Earlier studies (Dye 1974; Spiers 1985) indicated that basidia and basidiospore production require light and that spore release was optimum at 18°C (Spiers 1985). For 24 hours, the spores were cast onto 1.25% MEA (with the exception of one test on water agar), identical medium to that upon which the crystals were first observed. The plates were incubated in the growth chamber and observed periodically for the development of crystals. One test using water agar was conducted with source JAM-7.

Collecting the crystals. Due to the light-weight nature of the crystals it was necessary to collect a large amount in order to yield enough for testing. Initial tests demonstrated that the crystals were soluble in ethanol but not water, therefore a purification procedure involving dissolving the crystals and filtering the solution was devised.

Crystals and some of the surface of the mound of spores from which they had emerged were scraped off with a scalpel. The scrapings were placed in 95% ethanol. The mixture was then filtered through a 2.0 μm filter to remove any mycelium, spore casings, or medium which may have been included in the scrapings. The resulting solution was evaporated off, leaving a mat of reformed crystals in the bottom of the beaker. De-ionized water was added to the

beaker and left overnight to dissolve any sugars or other water-soluble substances which may have been dissolved by the ethanol. The resulting suspension of crystals was filtered through a 2.0 μm filter and the crystals collected from the filter. The crystals were again dissolved in 95% ethanol and filtered through a 0.2 μm filter to assure that all bacteria or other particulate matter were removed. The solution was then evaporated off, leaving a mat of crystals in the beaker.

Another recrystallization technique was also occasionally employed. The solution was heated in a 10 ml sample jar on a laboratory hotplate at low heat. The compound sublimated on the walls of the beaker, forming erect, needle-like crystals identical to those that formed on the agar surface.

Crystal morphology. Crystal morphology was studied using dissecting and phase-contrast light microscopy and with scanning electron microscopy (SEM) on a Hitashi 570 Scanning Electron Microscope.

Instrumentation tests. A variety of tests were conducted to determine the properties of the crystals. These included elemental analysis and x-ray microanalysis, mass spectroscopy, powder x-ray diffraction (XRD), FT/Infra Red (IR) and FT/Nuclear Magnetic Resonance (NMR) spectroscopy, and a melting point test.

Crystalline material (purity unknown) was analyzed with a CEC 240-XA Elemental Analyzer to determine the carbon, hydrogen, nitrogen ratio. A test for inorganic elements was also conducted. X-ray microanalysis of un-coated crystals was made with a Hitashi 570 SEM with a Tracor Northern 5502 energy dispersive x-ray spectrometer (limited to detecting elements with atomic No. 11 or greater). Accelerator voltage was 20 kv and the acquisition time was 100 sec.

A Hitashi RMU-6E Mass Spectrometer was used to determine the molecular weight of the crystal compound. The analysis was done at both 12.5 e.v. (ionization potential) and 80 e.v.

A powder XRD pattern was obtained with a Debye-Scherrer camera mounted on a Philips x-ray generator.

FT/IR spectroscopy was conducted with a Bruker IFS 66 FT/IR spectrometer with an attached microscope in transmission mode.

A basic proton (i.e.H^1) spectrum (0 ppm - 10 ppm) and a basic broad band decoupled C^{13} spectrum were obtained on a Bruker AC200 FT/NMR spectrometer.

The melting point of the crystals was recorded on a Gallenkamp Melting Point Apparatus.

Results

Crystal production. Basidiocarps from all 13 test strains produced crystals on MEA but some strains were more reliable and prolific producers than others. Basidiocarps from the strains JAM-7, JAM-10, and JAM-11 were the most reliable and productive. JAM-7 and JAM-10 began producing crystals 10-12 days after the spores were cast. JAM-11 was the fastest of all strains, consistently producing crystals after only 4-5 days. The other strains were either not as prolific, slower, or less reliable compared to the above three sources (Table 3.1). JAM-7 was also tested on water agar. No crystals formed on this medium.

The duration of crystal development was limited. Growth and spread continued for one to two weeks after they first appeared.

Table 3.1. Basidiocarp strains and crystal production.

Source	Days from spore cast until crystal growth	Crystal Production Rating
JAM-1	14	good
JAM-2	14	excellent/poor [a]
JAM-7	10-12	excellent
JAM-10	10-12	excellent
JAM-11	4-5	excellent
LU-9	14-18	poor
LU-13	14	poor
Aspen	21	poor
Emmerson-2	16	poor
Harrington	72	poor
Isabella	14	good
Woodlot	14	fair
Oliver	18	poor

[a] twice very productive; almost no crystals another time

No crystal compound was extracted from spore casts which had not produced crystals.

Crystal morphology. At the macroscopic and low-magnification level the crystals appeared as mono-filamentous needles of various diameters, sometimes exceeding 1 cm in length (Figs. 3.1, 3.2). They arose from the surface of the dense spore cast as either single needles or in clusters, the clustered needles radiating out from a common point of origin. Microscopy revealed that the crystals were actually bundles of individual filaments composed of even finer crystal strands. Evidence of both filamentous bundle structure and a striated sheet-like structure is apparent in the SEM micrographs (Figs. 3.3, 3.4).

Instrumentation test results. Calculations and details pertaining to the tests are presented in Appendix III.

Elemental analysis/X-ray microanalysis - A preliminary elemental analysis for carbon, hydrogen, and nitrogen estimated that the crystal compound was made up of 76.9% C (+/- 0.5%), 11.33% H (+/- 0.3%), and 0.15% N (+/- 0.2%) by weight. X-ray microanalysis did not detect inorganic elements in the crystals, within the machine's detection limits (0.05%).

Mass spectroscopy - Mass spectroscopy showed a peak at 222 (M^+), with an intense peak at 204 (M^+-H_2O). At 80 e.v. the largest peak was at 161 (M^+-61). An unsuccessful attempt

to match the peak pattern characteristics with those of known compounds listed in the Wiley-NBS library was made. On the basis of elemental analysis, biogenetic considerations, and mass spectroscopy, a molecular formula of $C_{15}H_{26}O$ is proposed for the compound (D. Orr pers. comm.).

XRD - An XRD pattern was obtained (Appendix III) but an attempt to match the pattern with those catalogued in the JCPDS Powder Diffraction File - 1988 (PDF-2 Database Sets 1-38, JCPDS-ICDD 1988 Release D6) was unsuccessful.

FT/IR - The IR spectrum (carried out on a single crystal) showed absorption at 3300 cm^{-1}, suggesting the presence of a hydroxyl group. There was no absorption in the carbonyl region of the spectrum.

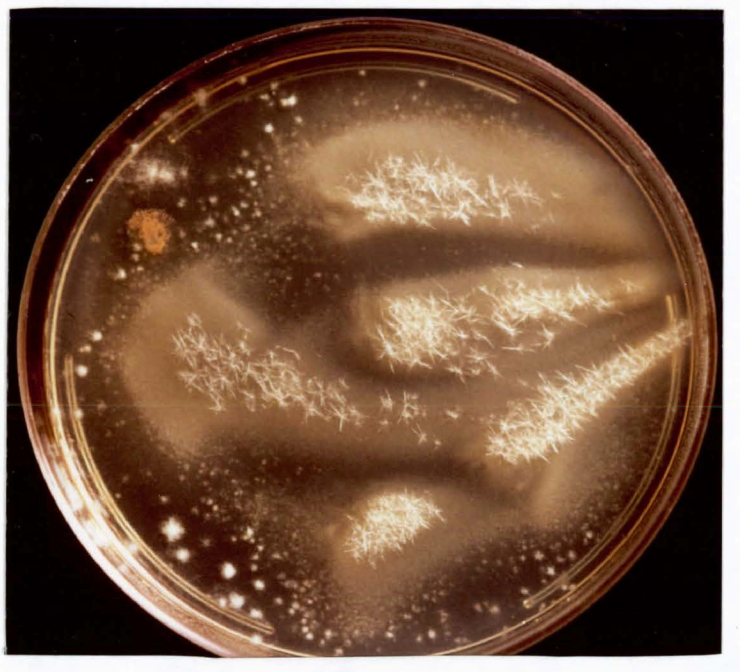


Figure 3.1. Needle-like crystals arising from dense spore casts of *C. purpureum*.

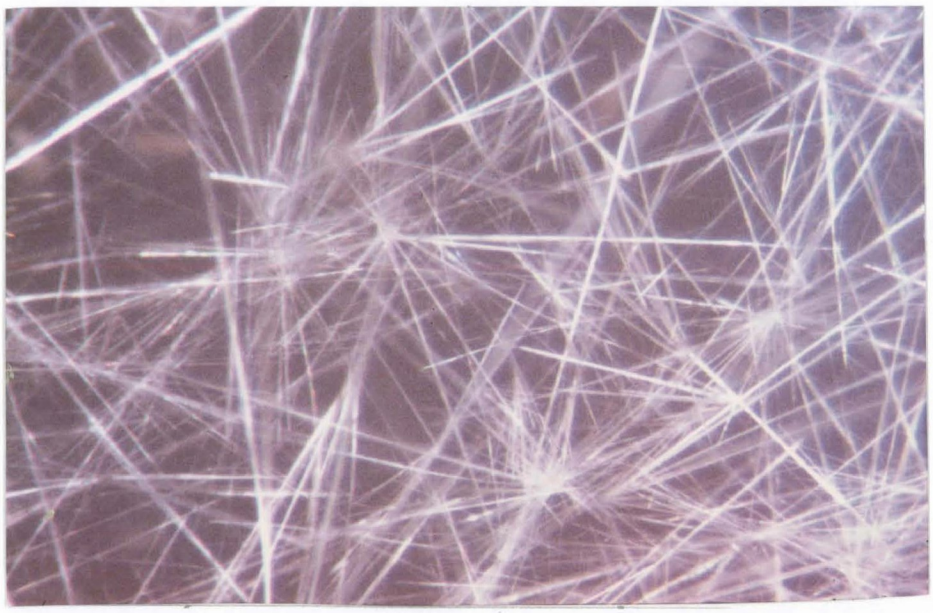


Figure 3.2. Needle-like crystals - various lengths and diameters. x 12

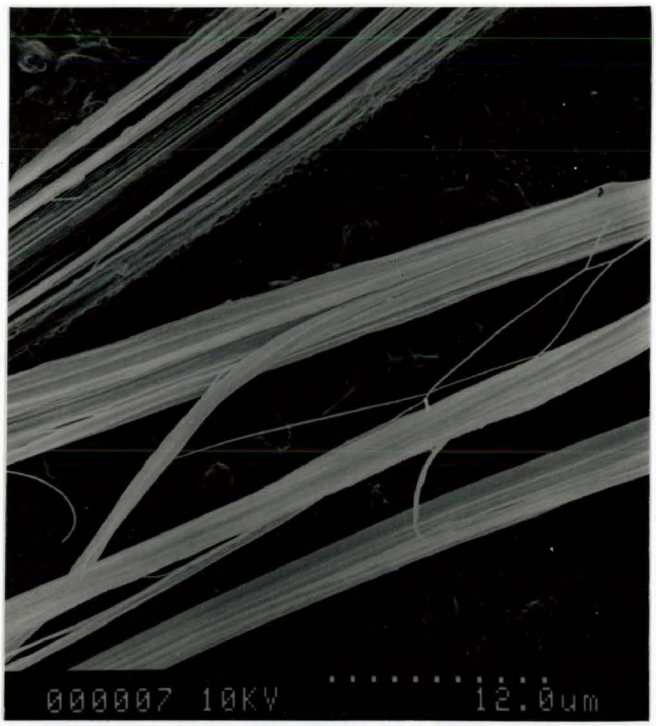


Figure 3.3. SEM micrograph showing filamentous bundle structure.

Figure 3.4. SEM micrograph showing striated, sheet-like structure of crystals.

FT/NMR - The H^1 and C^{13} NMR spectrums indicate a compound which is mainly hydrocarbon in nature and which contains four methyl groups, possibly an oxygen atom, and also a double bond carbon pair (see Appendix III).

Melting point - The crystals melted at approximately 135-136^{o}C in a sealed glass tube. They began to sublime at approximately 130^{o}C.

Discussion

Origin of crystal compound. The molecular formula $C_{15}H_{26}O$ identifies the crystal compound as a sesquiterpene (i.e. a compound derived from three isopentane units). The

sesquiterpenes are secondary metabolites derived from the condensation of the primary metabolite, acetyl-CoA, in the mevalonic acid pathway, with farnesyl pyrophosphate as the immediate precursor (Goodwin & Mercer 1972).

In this study, the combination of a carbon-rich medium such as 1.25% MEA and the nutrient demands of millions of germinants favoured production of secondary metabolites. There are several elements (e.g. phosphorus, nitrogen, zinc) and vitamins (e.g. thiamine), which are, along with carbon, essential for growth. Exhaustion of any of these essential elements in the presence of excess carbon can trigger production of secondary metabolites (Griffin 1981).

Identifying the limiting factor which caused a shift of metabolic activity from a primary into a secondary pathway can be difficult because of the diverse nutrient requirements of the fungi (Gottlieb 1978). No exogenous nutrients are required for germination of *C. purpureum* spores (Beever 1969), therefore, the limiting factor must be a nutrient required for subsequent growth of the germinant hyphae. In a test of the effects of seven vitamins on the growth of *C. purpureum*, Beever (1969) found only thiamine necessary. Further study of the mechanism triggering the production of the crystal compound should, therefore, begin with tests on the effect of thiamine levels on crystal production.

Inter-strain variation in crystal production. There was significant variation in crystal production among strains, especially in the length of time from spore cast to the initial appearance of crystals. Variations in secondary metabolite production can depend on the genetic constitution of the fungal strain, as has been shown for penicillin production (Turner 1975).

In this study, crystal production habits were consistent with strain. For example, strain JAM-11 consistently produced many crystals starting 4-5 days after spore cast, whereas JAM-7 required 10-12 days. This individuality is most easily interpreted as a manifestation of genetic variation among strains.

If production of the crystal compound is triggered by depletion of an essential nutrient such as thiamine (a coenzyme) or nitrogen, variation may simply be due to different growth rates among strains and therefore overall more rapid utilization of all nutrients, including the limiting factor. Another possibility is that a strain may have a greater demand for a particular nutrient, thus depleting it sooner than other strains. It is also quite possible that much more complex genetic and biochemical processes are at work.

The observed limited duration of crystal formation is consistent with the correlation between secondary metabolism and developmental stage, a phenomenon widely

observed (Demain 1972). Cessation of crystal production may signalize transition from one stage of hyphal development to another. It may, however, also indicate that another limiting factor has been exhausted.

Secondary metabolites of *Chondrostereum purpureum*. The best known secondary metabolite of *C. purpureum* is a pectinase (endopolygalacturonase I (EC 3.2.1.15)) which induces the silver-leaf symptoms on fruit trees (Miyairi *et al.* 1985). Brooks and Brenchley (1929) found evidence of another mycotoxin which killed leaf and flower tissue in Victoria plum. This mycotoxin has not been identified.

More recently, Ayer *et al.* (1981) isolated from *C. purpureum* a unique group of sesquiterpenes which they named sterpurenes. Several sterpurenes have been identified, including the parent hydrocarbon sterpurene, sterpuric acid, sterpurene-3,12,14-triol (Ayer *et al.* 1984), and 9,12-dihydroxysterpurene (Abell & Leech 1988). The sterpurenes are characterized by a tricyclic carbon skeleton which includes a cyclobutane ring.

Sterpurene, precursor of the oxygenated metabolites (e.g. acids), is derived from farnesyl pyrophosphate via humulene (Murata *et al.* 1981; Ayer *et al.* 1984). This biosynthetic pathway is the route by which many sesquiterpenoid metabolites of fungi of the class Basidiomycetes arise (Ayer & Browne 1981).

The sterpurenes, like many fungal secondary metabolites, may be toxic (Griffin 1981), but they have not yet been linked to any pathological symptoms.

Identification of the crystal compound. The sesquiterpenoid crystal compound and the sterpurenes probably arise along a basically identical biosynthetic pathway. In addition, sterpurene-3,12,14-triol forms white needle-like crystals that melt at 146-148^{o}C (Ayer & Saeedi-Ghomi 1981), only 10 degrees higher than the crystals of this study. Based on these similarities, and the isolation of an increasing number of sterpurene compounds from cultures of *C. purpureum* (Abell & Leech 1988), there appears to be reason to conclude that the crystal compound is a sterpurene, but one with properties (e.g. melting point, molecular weight) unlike any other described in the literature.

There is, however, evidence which excludes the crystal compound from the sterpurene group. The mass spectrums of compounds with the sterpurene skeleton all show a significant peak at M^{+} -28 (i.e. $-C_2H_4$). The loss of ethylene (C_2H_4) through cleavage of the cyclobutane ring is characteristic of the sterpurenes. This peak was totally absent in the mass spectrum of the crystal compound of this study.

Devon and Scott (1972) list 66 sesquiterpenes with the molecular formula $C_{15}H_{26}O$ and molecular weight 222, some

oils and some solids. Only three bicyclic tertiary alcohols, α-verbesinol, g-cadinol, and (+/-)-torreyol (syn.= d-δ-cadinol) are similar enough to be considered serious possibilities. α-Verbesinol is a crystalline isomer derived from an alcohol extracted from the roots of *Verbesina virginica* L. (Gardner *et al.* 1961). Its molecular weight, molecular formula and melting point (134-136°C) match those of the crystal compound from *C. purpureum*. g-Cadinol, extracted from eucalyptus oil (McKern *et al.* 1954), also matches the crystal compound in molecular formula, molecular weight and melting point (Devon & Scott 1972).

(+/-)-Torreyol (also known as cadinol) is a compound isolated from numerous sources (Borg-Karlson *et al.* 1981), including several conifer species (Westfelt 1966). The levorotatory form (i.e. (-)-torreyol) has been isolated from turpentine derived from kraft pulping of *Pinus silvestris* L. (Borg-Karlson *et al.* 1981). White crystalline needles of the dextrorotatory form (i.e. (+)-torreyol) are produced by cultures of the fungi *Clitocybe illudens* Schw. (Nair & Anchel 1973) and *Stereum hirsutum* (Willd.:Fr.) S.F. Gray (Ainsworth *et al.* 1990; Rayner 1991). Torreyol has also been identified in the wings of the male butterfly *Lycaeides argyrognomon* (Bergstrasser) where it, along with other volatile compounds, plays a role in courtship behaviour (Lundgren & Bergstrom 1975).

Absence of IR, NMR and mass spectrums for α-verbesinol and g-cadinol prevent detailed comparison of these compounds with the *C. purpureum* crystal compound. However, the close similarity of the ring structures of g-cadinol and (+)-torreyol (Devon & Scott 1972) suggests that g-cadinol is a form of torreyol. Likewise, the spectrums of (+/−)-torreyol (Rodriguez-Avial Franke *et al.* 1984; Ainsworth *et al.* 1990) and the *C. purpureum* crystal compound (see Appendix III), although not identical, are also similar.

More investigation by organic chemists is required in order to determine definitely whether the crystal compound is a new discovery or a previously described compound not yet reported for *C. purpureum.* This research should explore the relationship of the *C. purpureum* crystal compound to the torreyols.

Further, to test the compound for phytotoxic effects similar to those observed by Brooks and Brenchley (1929) it would be necessary to introduce the compound into the transport tissue of trees. However, in its crystalline form the compound is not soluble in water. Such a test would be complicated by confounding effects arising from the probable phytotoxic effect of the organic solvent (e.g. ethanol) needed to dissolve the compound. Further work must be done to determine a suitable non-toxic solvent or non-toxic concentration of solvent.

A Study of the Sexuality of *Chondrostereum purpureum*

Few studies have been conducted on the sexuality of *Chondrostereum purpureum*. Early studies by Kniep (1920) and Robak (1936, 1942) indicated that *C. purpureum* is heterothallic, with tetrapolar sexual differentiation. This characteristic supports Pouzar's (1959) reclassification of the species from the genus *Stereum*, which is generally considered homothallic (Welden 1971), to a new, monotypic genus, *Chondrostereum*.

Fruiting bodies of tetrapolar species produce homokaryons of four mating types. Homokaryon progeny are self-sterile, intersterile (i.e. incompatible) with their type and with two other types, and cross-fertile (i.e. compatible) with only one other type (Raper 1966). Hyphae of compatible mating types fuse and produce dikaryons (i.e. heterokaryotic dikaryons) which are controlled by two factors, the A and B factors. Nuclear association, conjugate division, and clamp initiation are under the control of the A factor. The B factor controls nuclear migration and clamp fusion. The A and B factors may be comprised of two linked loci, A_α and A_β, and B_α and B_β (Dick 1965), or of several loci, each with two or more alleles (Papazian 1951). Compatibility between homokaryons exists when they possess unlike A and B factors.

The objective of this study was to determine the sexuality of a strain of *C. purpureum* isolated from white birch in Thunder Bay, Ontario.

Materials and Methods

A sporulating basidiocarp from the source JAM-7 was attached to the underside of a petri dish lid. After 24 hours, germinants were lifted from the agar surface with a pin point and transferred into petri dishes. Successful transfers were grown for 1 week and then stored in the refrigerator. Twelve single-spore isolates were paired in petri dishes in all combinations (66 pairs). Before pairing, the single-spore isolates were microscopically examined to confirm the absence of clamps.

After incubation for 2 weeks, mycelium from the zone of confluence was microscopically examined for the presence of clamps (Boidin 1986). The outer margin associated with each isolate was likewise examined for clamps in order to detect possible unilateral compatibility.

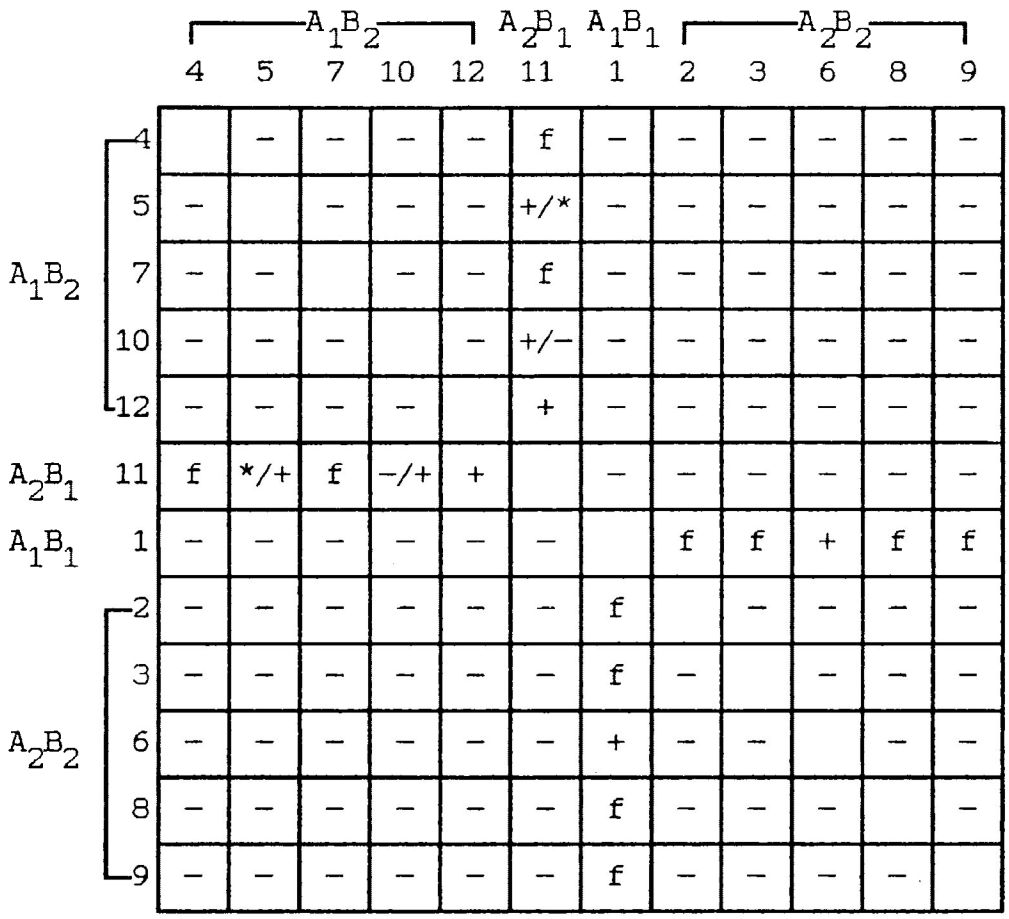
The pairings were incubated for an additional 6 weeks to see if fruiting would occur. After this further incubation period, each successfully mated pair was examined to determine the relative abundance of clamps and fruiting in each of the single-spore isolates. In plates where fruiting had occurred, mycelium approximately 5 to 10 mm from the developing hymenial surface was examined. Where no fruiting had occurred, mycelium was sampled from midway between the inoculation point and the edge of the plate. Relative abundance of clamps was measured as the number of clamps observed in 5 minutes.

Macroscopic interaction characteristics of isolate pairs were also recorded. Typical characteristics include development of an aversion zone ("barrage reaction") between the pairs, reduced growth of aerial hyphae ("flat reaction"), hyphal massing at the confluence zone ("barrier reaction"), and no reaction, where the cultures are essentially indistinguishable from homokaryons (Raper 1966; Wilson 1990).

Results

Clamp formation. Clamps formed in 10 of the 66 pairings (Fig. 4.1). Clamps were abundant in some pairings but rare in others. In two cases compatibility appeared unilateral (i.e clamps formed in only one isolate of the pair) when the plates were examined after 2 weeks growth. In one of these cases (i.e. pairing 5,11), however, clamps were observed in both isolates when the pairing was 8-weeks-old.

The pattern of successful matings showed that the 12 isolates from JAM-7 were distributed into four mating types, A_1B_1, A_1B_2, A_2B_2, A_2B_1, in the proportion 1 : 5 : 5 : 1 respectively.



+ = successful bilateral mating (compatible)
- = unsuccessful mating (incompatible)
f = clamps and fruiting observed
+/*, */+ = Clamps were observed in isolate No. 5 only after eight weeks growth.
-/+, +/- = Clamps were observed only in isolate No. 11.

Figure 4.1. Mating reactions of single-spore isolates of *C. purpureum*.

Fruiting. Fruiting occurred in six of the 10 plates where the isolates had mated and formed clamps (Table 4.1). Fruiting occurred in four of the five A_1B_1 X A_2B_2 pairings and was always more abundant on the A_1B_1 side (Fig. 4.2a). Two of the five A_1B_2 X A_2B_1 pairings fruited, in one case (i.e. pair 4,11) only on the A_2B_1 side, but in the other case (i.e. pair 7,11) on both sides (Fig. 4.2k). The A_1B_1

X A_2B_2 fruiting (Figs. 4.2a, 4.3) was typical of fruiting normally observed (i.e. cream-coloured mounds sometimes widespread but primarily along the side of the petri dish, eventually merging into a more-or-less continuous rind of fruitification (van der Westhuizen 1958)). Fruiting in A_1B_2 X A_2B_1 pairings differed from normal. Cream-coloured hymenial structures often developed as elongated, stringy growths rather than forming typical individual mounds (Figs. 4.2k, 4.4). No fruiting occurred in plates where clamps were absent.

Table 4.1. Relative abundance of clamps and fruiting after 8 weeks.

	First Isolate of Pair		Second Isolate of Pair	
Isolate Pair	# of Clamps Observed	Fruiting	# of Clamps Observed	Fruiting
A_1B_1 X A_2B_2				
1,2	1	abundant	11	minimal
1,3	1	abundant	9	minimal
1,6	1	none	42	none
1,8	1	moderate	2	minimal
1,9	6	abundant	17	minimal
A_1B_2 X A_2B_1				
4,11[a]	4[b]	none	4	abundant
5,11[a]	6[b]	none	28	none
7,11[a]	6	abundant	16[c]	abundant
10,11	0[c]	none	7[c]	none
12,11	5[c]	none	5[c]	none

[a] - the hyphae on the 11-side was unusually thick-walled
[b] - no clamps had been observed on the 5-side after 2 wk
[c] - numerous false clamps were also observed

Macroscopic interactions. Several types of macroscopic cultural interactions were observed between the single-

spore isolates. The reactions in the uncommon-AB matings differed between A_1B_1 X A_2B_2 matings and A_2B_1 X A_1B_2 matings. In the A_1B_1 X A_2B_2 matings both cultures were similar in appearance with only a slight sign of reaction between them in the confluence zone (Fig. 4.2a). In the A_1B_2 X A_2B_1 matings no reaction zone was evident and the growth in the A_2B_1 culture was more aerial, especially around the inoculum plug (Figs. 4.2f, 4.2k).

Similarly to the uncommon-AB matings, the common-A matings reactions varied. The A_1B_1 X A_1B_2 matings blended with little noticeable reaction in the confluence zone and both cultures were similar in appearance (Fig. 4.2b). The A_2B_2 X A_2B_1 matings exhibited more aerial growth and some reduced growth in the confluence zone (Fig. 4.2e).

Hyphal massing at the confluence zone or extensive aerial growth over only one culture of the pair characterized 24 of the 26 common-B matings. Barrage reactions of various intensities also were commonly observed (Figs. 4.2c, 4.2d).

In 13 of 20 common-AB matings, the two cultures were very similar and barrage reactions of various strengths were distinguishable in the confluence zone (Figs. 4.2g, 4.2h). Two of the remaining seven were identical in appearance and had grown into each other without a noticeable reaction at the confluence zone. Of the remaining five, one of each pair was either a denser or more aerial culture, and in three of the pairs a barrage reaction was evident.

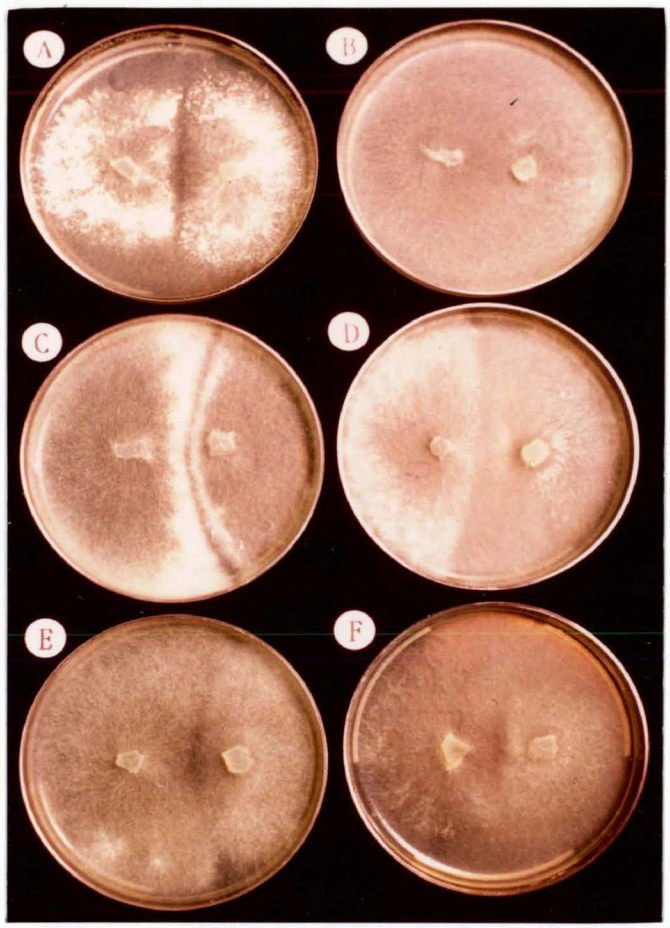


Figure 4.2. Macroscopic cultural interactions between paired single-spore isolates of *C. purpureum* after 2 wk. **A.** uncommon-AB (A_1B_1 X A_2B_2) **B.** common-A (A_1B_1 X A_1B_2) **C.** common-B (A_1B_1 X A_2B_1) **D.** common-B (A_2B_2 X A_1B_2) **E.** common-A (A_2B_2 X A_2B_1) **F.** uncommon-AB (A_1B_2 X A_2B_1).

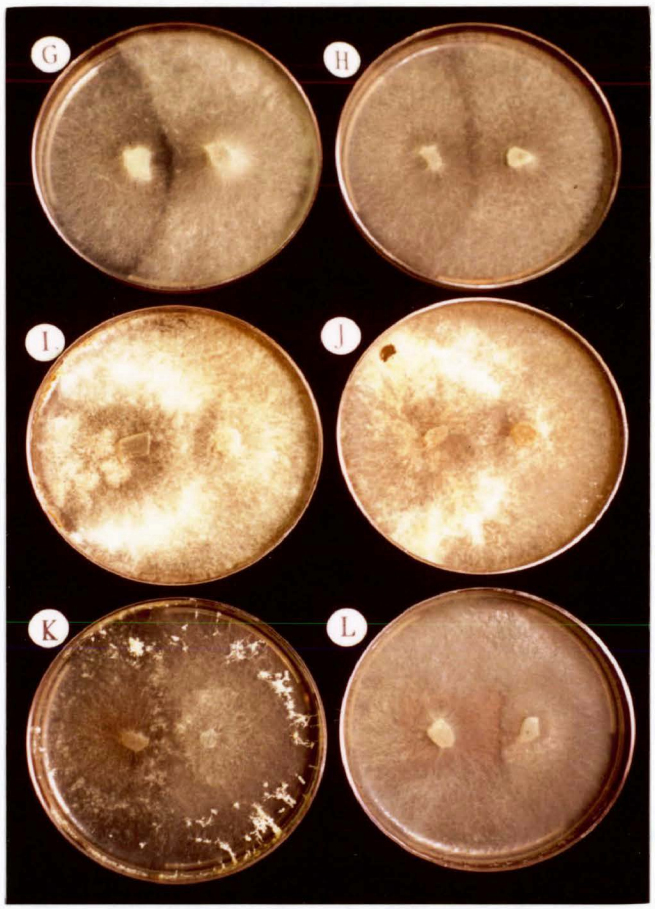


Figure 4.2 (cont'd). G. common-AB (A_2B_2 X A_2B_2) H. common-AB (A_1B_2 X A_1B_2) I. & J. uncommon-AB (A_1B_1 X A_2B_2) after 5 wk, with and without fruiting, respectively K. & L. uncommon-AB (A_1B_2 X A_2B_1) after 5 wk, with and without fruiting, respectively.

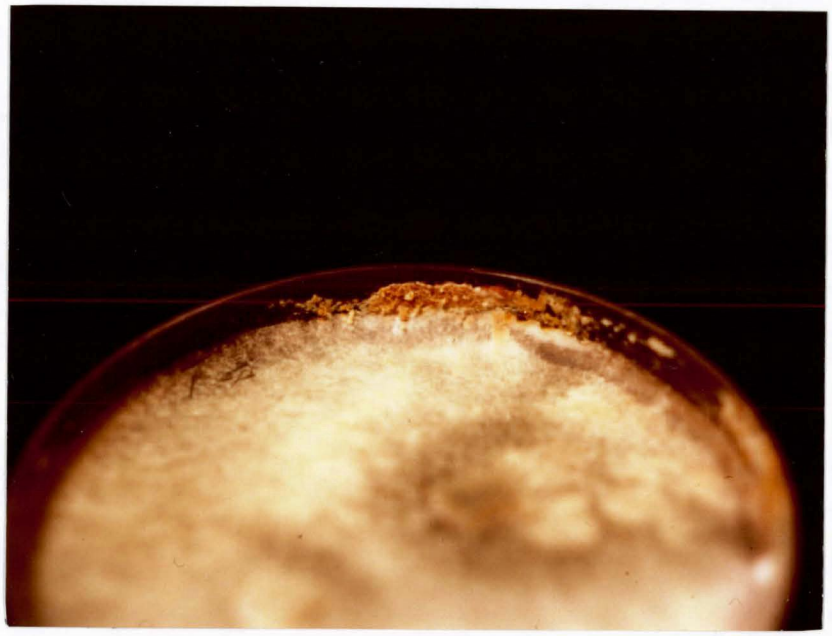


Figure 4.3. Fruiting in culture normally observed with *C. purpureum* (A_1B_1 X A_2B_2 mating).

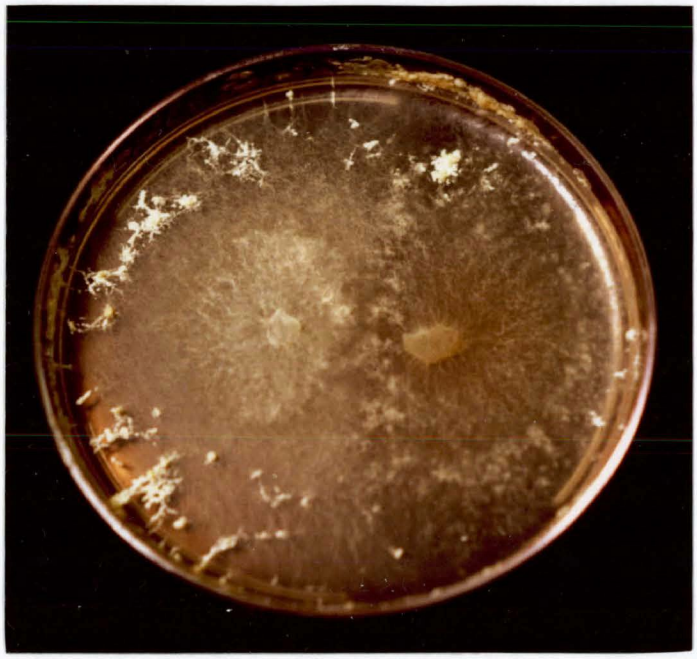


Figure 4.4. Unusual "stringy" fruiting in culture (A_1B_2 X A_2B_1 mating).

Discussion

Robak's (1936, 1942) reports on the sexuality of *C. purpureum* indicated a tetrapolar differentiation of sex. The current study confirms Robak's results but encountered factors unreported by him. Interesting observations include: far fewer clamps in the A_1B_1 isolate than in the A_2B_2 isolate in A_1B_1 X A_2B_2 matings, delayed production or absence of clamps in the A_1B_2 isolate of two A_1B_2 X A_2B_1 positive matings, thick-walled hyphae and both false and true clamps in some A_1B_2 X A_2B_1 pairs, unusual fruiting in the A_1B_1 X A_2B_2 matings, and delayed clamp formation and fruiting in pairs (i.e. two common-AB pairs) which had been deemed incompatible at an earlier (2-week-old) stage (Table 4.1).

Raper (1966) stated that tetrapolar species produce four mating types in equal frequency. The 12 single-spore isolates paired in this study represented four mating types distributed disproportionately into four mating types (i.e. 1 : 5 : 5 : 1). Robak (1942) conducted tests with 10 and 14 isolates which were distributed in the proportions 2 : 2 : 2 : 4, and 4 : 2 : 7 : 1, respectively. Additional studies with larger numbers of single-spore isolates would be required in order to determine whether the theoretical equal distribution of mating types described by Raper applies to this species.

Clamp formation. In positive matings, nuclei migrate from the mate through the existing hyphal system until they reach growing tip-cells where they establish dikaryons with the resident nuclei. Subsequent hyphal growth includes conjugate division of the two nuclei and migration of a daughter nucleus from each of the dividing nuclei (Raper 1966). This condition would suggest that, after successful mating, the paired isolates would contain identical dikaryotic cells and would develop in a similar manner. This was not the case with the A_1B_1 X A_2B_2 matings. In all five A_1B_1 X A_2B_2 matings, clamp connections were more abundant in the A_2B_2 isolates than in the A_1B_1 isolate. Clamp connections were also more abundant in the A_2B_1 isolate than in the A_1B_2 isolates in three of five A_1B_2 X A_2B_1 matings (Table 4.1).

It is unlikely that differences in the B factors alone account for this consistent difference. In the A_1B_1 X A_2B_2 matings the nuclei with the B_2 factor migrated into the clamp-poor A_1B_1 isolate whereas in the A_1B_2 X A_2B_1 mating the nuclei with the B_1 factor migrated into the clamp-poor A_1B_2 isolate. If migration competence of the B_2 and B_1 factors was the principal determinant of clamp abundance the A_1B_1 and A_1B_2 isolates should not have been similarly clamp-poor in relation to their mates.

A more comprehensive approach to nuclear migration is Rayner *et al.*'s (1984) proposed three basic phases

underlying the formation of stable mating-type heterokaryons: access migration, acceptor migration, and stabilization. According to this scheme, observed interaction types can be accounted for as either independent or combined expressions of these phases. In access migration, passage of the donor nucleus occurs at a rate dependent on the genetic relationship between the donor and recipient nuclei. Asymmetric or unilateral patterns of nuclear migration can result. In acceptor migration, the migration rate of donor nuclei is controlled solely by the acceptor mycelium; thus, nuclear migration would occur at the same rate, regardless of which other mating type was involved. Ultimately, either of these migration phases can lead to the stabilization phase, where sustained co-existence of nuclei of different mating type is attained.

Another possibility is that although the nucleus-pairs in the dikaryotic cells on the margin of each culture are identical, they may not function identically. There may be a dominance exercised by either the host or donor nucleus. Thus, the A factor of either the host or the donor may control the amount of clamp initiation that occurs.

The literature indicates the clamp forming behaviour is non uniform in all strains of *C. purpureum*. Chamuris (1988), Boidin (1971) and Stalpers (1978) each described different clamp forming behaviour in this fungus. These

studies were probably conducted using older dikaryotic cultures. If the host or donor A factor does exert dominance over clamping behaviour, an older culture would express an average clamp-forming competence that reflects the competence of the monokaryon isolates from which it was derived. This study provided an opportunity to observe individual clamp-forming competence of such component monokaryons.

These hypotheses could be tested by mating the A_1B_1 and A_2B_2 isolates with A_3B_3, A_4B_4, etc. isolates from other basidiocarps. If identical clamp-forming competence is observed in the A_1B_1 and A_2B_2 isolates as was originally observed, evidence supporting the acceptor migration or A factor host-dominance hypotheses would be provided. Different clamp-forming competence would support the access migration or donor-dominant hypotheses. This test could also be applied to the A_1B_2 and A_2B_1 isolates.

Earlier work by Brodie (1948) and Raper and Miles (1958) investigated unilateral dikaryotization, i.e. the failure of reciprocity of nuclear exchange. This phenomenon is often correlated with morphological mutant strains which act as donors, but not receptors of nuclei (Raper and Miles 1958). Lack of reciprocity may explain the absence of clamps in the No.10 isolate of the pair 10,11 and the delayed clamp production in isolate No.5 in pair 5,11. The morphological peculiarities (i.e. appressed growth in the

A_1B_2 isolate, denser growth around the A_2B_1 inoculum plug, thick-walled cells in the A_2B_1 isolate) associated with the A_1B_2 X A_2B_1 matings also support the hypothesis that the A_1B_2 mating type is aberrant. Rayner *et al.*'s (1984) access migration phase concept would also explain the delayed or absent clamp formation observed. In addition, the appressed growth in the A_1B_2 isolates is a mycelial morphological feature associated with access migration.

The presence of both true and false clamps in some of the A_1B_2 X A_2B_1 cultures suggest that both common-B and uncommon-B interactions have taken place. These may be due to mutations in the B factor (Raper 1966), to an extraneous cause such as contamination (Whitehouse 1949), or to asexual recombination of genetic material (parasexuality) in the hyphae (Griffin 1981).

Fruiting. Fruiting in culture is common with this fungus. Robak (1942) reported that a Canadian strain of *C. purpureum* was generally very fertile in culture and that pairings which did not fruit exhibited mycelial "concretions" which he interpreted as abortive hymenia.

Numerous factors affect fruiting. Accumulation of reserve food materials, stimulation by light, aeration (Raper 1966; Dye 1974), accumulation of waste products or staling (Kendrick 1985), nitrogen depletion, availability of thiamine (Wessels 1965) and temperature (Hawker 1966), are all external factors linked to initiation and

development of fruiting bodies. In addition, internal biochemical factors interact with external factors. Gene activation or repression of enzymes related to sporulation mechanisms have been demonstrated (but links to specific genes have not been made) (Griffin 1981).

While fruiting in the A_1B_1 X A_2B_2 matings appeared typical (i.e. of JAM-7 fruiting in culture) it mostly occurred in the A_1B_1 isolate. This result suggests differential fruiting competence between the two mating types.

Raper and Krongelb (1958) found that fruiting timing and abundance varied widely in *Schizophyllum commune* Fr. and that early and abundant fruiting was not random, rather, it was always associated with dikaryons containing specific strains. Late or poor fruiting was also associated with specific strains which were sometimes components of early-fruiting dikaryons. From these results they hypothesized that fruiting competence was genetically determined and that high competence dominates low competence. Subsequent tests confirmed the first hypothesis but demonstrated that fruiting competence is inherited as a polygenic character and not as a simple character with dominant and recessive alleles.

As in the case of clamp-formation competence being the result of difference in A factors, the question of fruiting competence as related to host or donor nuclei remains

unanswered. Similarly, this could be tested with matings with mating types from other basidiocarps.

The delayed production of clamps and fruiting in isolate pairs 3,8 and 3,9 is problematic. All three isolates mated successfully with isolate No.1 (A_1B_1), indicating compatibility due to different mating type (A_2B_2). As with the false clamps found in the A_1B_2 X A_2B_1 matings, these anomalies may be due to mutations, an extraneous cause such as contamination, or to asexual recombination of genetic material (parasexuality) in the hyphae.

Macroscopic interactions. Interacting mycelia of the paired isolates exhibited a variety of macroscopic characteristics. These characteristics were similar to those described by Wilson (1990) for *Echinodontium tinctorium* (Ell. & Ever.) Ell. & Ever. but there were some differences in their distribution among the mating-type combinations.

Compatible pairings (uncommon-AB) grew together with little sign of reaction in the confluence zone, similar to Wilson's observations. Common-A interactions, likewise were similar to those observed by Wilson, i.e. not consistent. This observation differs, however, from other studies (Raper 1966) where common-A isolates were typically sparse and appressed.

The dominant characteristic of common-B interactions in the current study was hyphal massing (i.e. barrier

reaction) or extensive mycelial aerial development. The barrage reaction commonly associated with common-B matings (Raper 1966) also was observed. While the appearance of a barrage reaction in only some of the pairings is similar to observations made by Wilson (1990), the predominance of extensive aerial growth and barrier reaction is not mentioned in other reports (Wilson 1990; Raper 1966) of common-B matings.

Interactions reported of common-AB matings vary. They include no reaction (Raper 1966), where both isolates grow together without discernible reaction, barrier reaction (Wilson 1990), and barrage reaction (Wilson 1990). The barrage reaction was the main interaction observed in this study. Unlike Wilson's study of in *E. tinctorium*, no barrier reaction occurred between the common-AB isolates.

This study raised more questions than it answered about the incompatibility system of the sexuality of *C. purpureum*. Follow-up research is needed to investigate the clamp-formation and fruiting anomalies observed, as well as the distribution of mating types. These studies will undoubtedly raise even more questions because the fungal incompatibility system is "complex in its genetic structure, in its physiological and morphological manifestations, and doubtlessly in its biochemical operation" (Raper 1965).

Induced-Growth and Storage of *Chondrostereum purpureum* Basidiocarps

Chondrostereum purpureum has drawn renewed attention recently. It has been found associated with dieback and decline of urban white birch (McLaughlin & Setliff 1990) and is suspected as the primary agent causing so-called "post-logging decadence" of white birch (Setliff & McLaughlin in press).

Chondrostereum purpureum's potential as a silvicide also is being studied. In Canada, Wall (1990) demonstrated the potential of *C. purpureum* to reduce stump sprouting in several hardwood species, including red maple (*Acer rubrum* L.), sugar maple (*A. saccharum* Marsh.), yellow birch, white birch, trembling aspen, pin cherry (*Prunus pensylvanica* L.) and beech. In the Netherlands, de Jong *et al.* (1990a, 1990b) have investigated the efficacy of *C. purpureum* as a biological control for black cherry, and risks to non-target plants associated with the increased population of the fungus.

Current understanding of the ecology and pathogenicity of *C. purpureum* is limited. Few studies of its population structure have been made (Rayner & Boddy 1986). In addition, biochemical features of this fungus, such as the silvering agent (Miyairi *et al.* 1977, 1985) and the unique group of sesquiterpene metabolites (sterpurenes) (Ayer and Saeedi-Ghomi 1981) it produces raise many questions regarding its pathogenic mechanisms.

While many studies can be undertaken using cultures, others (e.g. spore production, sexuality studies) require live, sporulating basidiocarps. Studies may be hampered if researchers must rely on "wild" basidiocarps which may be at times difficult to obtain (e.g. during the winter). More often, a particular strain (especially those collected many years ago) is available only in culture.

The objectives of this study were to produce *C. purpureum* basidiocarps under controlled conditions and to determine the best storage method for live basidiocarps.

Materials and methods

Basidiocarp production. Six strains of *C. purpureum* were included in this study. They included the strains DAOM 21110 and DAOM 21818 received from the Canadian Collection of Fungus Cultures at the Biosystematics Research Centre in Ottawa. These strains had been stored *in vitro* as part of the collection since 1947 and 1949 respectively. A third strain, ECS 1695, had been collected in Thunder Bay, Ontario in 1987, while the remaining three strains, JAM-1, JAM-4, and JAM-5 were collected in Thunder Bay in 1989.

Lengths of sound, unstained white birch stem measuring approximately 30 cm long by 5 cm diameter were collected in the field and stored in a freezer. The stem segments were inoculated with *C. purpureum* according to a technique modified from Wall (1986). Pieces of white birch bark and

cambium (1 cm^2) cut from other samples were autoclaved and then placed in a petri dish at the margin of an actively-growing *C. purpureum* culture. After 16 days, the inoculated bark pieces were inserted into 1 cm^2 holes made in the bark at the midpoint of the stem sections. Five stem segments per strain were inoculated in this manner.

The inoculated stem sections were set on a greenhouse bench under natural light. Mist was applied to the stem sections (at a rate of 8 seconds of mist every 16 minutes) to keep them moist. The temperature on the bench was in the mid-twenties range throughout most of the study period but ranged from 15 to 36^{o}C at times. Basidiocarp production quantity and quality was assessed after two months. Limited trials with shorter (10-cm-long) stem sections and sections stood on end were also conducted.

Basidiocarp storage. Fresh basidiocarps were collected from source JAM-7. At the time of collection the basidiocarps were expanded and pliable but not fully saturated (dry *C. purpureum* basidiocarps are shrivelled and brittle). Four freezer storage treatments were tested: (1) not dried, in a glass sample jar, (2) air-dried, in a glass sample jar, (3) not dried, in a paper envelope, (4) air-dried, in a glass sample jar. Each treatment was replicated five times.

After 7 months in the freezer at -15^{o}C, the basidiocarps were thawed and soaked in water for 30 minutes, by which

time they were fully expanded. They were then attached with petroleum jelly to the underside of petri dish lids. The lids were placed over dishes containing 25 ml of de-ionized water to which three drops of the wetting agent Aerosol OT Solution (Fisher Scientific Company), had been added. The wetting agent was added to insure a more uniform distribution of spores in the water and therefore facilitate sampling. The dishes were put in a lighted (GE Chroma 50 fluorescent lamps) growth chamber set at 18°C.

After 9 hours, basidiospore release was estimated. Spores were counted using a Spencer Bright-Line Hemacytometer (American Optical Corporation). Five samples were taken from each dish. The viability of the spores cast was also tested. Spores were cast from each of the sporulating basidiocarps onto 1.25% malt extract agar and examined for germination success after 24 hours. At the conclusion of the experiment the area of the hymenial surface of each basidiocarp was measured using an Area Meter Mark 2 (Delta-T Devices Ltd.) and the rate of spore production per cm^2 was calculated.

Samples of the hymenium and the sub-hymenial layer of the basidiocarps were stained with phloxine and examined microscopically 5 days after the conclusion of the experiment. The condition of the hyphae and basidia was noted.

The mean spore release data could be analyzed with the Student-t test.

Results

Basidiocarp production. Basidiocarps were successfully grown in the greenhouse (Fig. 5.1). Basidiocarp production varied both among and within strains. Generally, the production level of the newer strains surpassed that of the older strains (Table 5.1).

Table 5.1. Basidiocarp production after two months.

Strain	# of productive stem sections	fruiting quantity	fruiting quality
DAOM 21110	3 of 5	few	poor/minute[a]
DAOM 21818	3 of 5	few	poor/minute[b]
ECS 1695	4 of 5	many	good/large[c]
JAM-1	5 of 5	few	poor-to-good
JAM-4	4 of 5	many	good/large
JAM-5	5 of 5	many	good/large

[a] Small, minimally developed basidiocarps or sometimes just a thin resupinate hymenial surface.

[b] One of these sections was later stood on end, raised slightly. It produced a well-developed hymenial surface over the lower end.

[c] Equalled or exceeded basidiocarps found in nature.

Figure 5.1. *Chondrostereum purpureum* basidiocarps grown on birch in the greenhouse.

Basidiocarp storage. The basidiocarps which had been frozen while wet, including those stored in glass and in paper envelopes, failed to produce spores. These basidiocarps had a dark, greasy, water-soaked appearance after soaking and lacked the firmness that characterized the air-dried basidiocarps.

Spore release by basidiocarps which were air-dried prior to freezing began less than 6 hours after they were removed from the freezer and soaked in water. Very little enlargement of the basidiocarps (evidenced by developing a pale mauve edge) occurred during the 9 hour duration of the experiment or the subsequent 24 hours.

Spore release by the air-dried basidiocarps which were stored in glass sample jars exceeded, in all five replications, the output by those stored in paper envelopes (Table 5.2). Spore germination after 24 hours from all samples was close to 100% and typical for this species.

Table 5.2. Effects of four storage treatments on spore release over 9 hours.

Treatment combination	Number of spores released (per cm^2 of hymenium)
frozen while wet, stored in glass	0
frozen while wet, stored in paper envelopes	0
air-dried before freezing, stored in glass	3 324 102 **
air-dried before freezing, stored in paper envelopes	1 890 580

** spore release by air-dried basidiocarps stored in glass greater (significant difference at $\alpha = .0025$) than by those stored in paper envelopes.

The hyphae and basidia of the air-dried basidiocarps were intact and appeared normal when hymenial and sub-hymenial tissue was microscopically examined 5 days after the experiment. The cell contents of the basidia absorbed phloxine stain, distinctly colouring the hymenial layer pinkish-red. The sub-hymenial layer was stained a light pink colour. In contrast, decomposition of the hymenial layer of the frozen-while-wet basidiocarps was evident. The hymenial layer was a diffuse, pale pink colour,

evidence that the basidia had lysed. The sub-hymenial hyphae were hyaline.

Discussion

Basidiocarp production. There was a distinct difference in fruiting competence between the old strains (i.e. DAOM 21110 and DAOM 21818) and the new strains, as evidenced by both the quantity and quality of fruiting (Table 5.1). This difference may be related to the growth and developmental rates of the old and new strains. The growth rate of wood-inhabiting Aphyllophorales which have been stored for long periods of time often decreases (Stalpers 1978). Moulds appeared to supplant *C. purpureum* in stem sections which produced poorly. A requisite for fruiting success was early monopoly of exposed wood surfaces, especially the ends of the stem sections.

Moisture level of the wood was also a critical determinant of fruiting success. Basidiocarps developed poorly, if at all, on very wet or dry portions of the wood. Uniformity and rate of misting, therefore, was a major factor in fruiting success variability within strains.

Overall, the method was successful, but with variable results due to factors discussed above. Two improvements can be made to the production technique to increase quantity and quality success. First, use shorter stem sections. *Chondrostereum purpureum* is not very competitive

with other early-succession fungi (Rayner 1978). The longer it takes for *C. purpureum* to reach the exposed wood surface, the greater the possibility for moulds to become established, thus preventing basidiocarp development.

The 30-cm-long stem sections were inoculated at the mid-point, therefore the fungus had to grow at least 15 cm through the wood before it reached an exposed wood surface where it would fruit (although fruiting sometimes developed at small bark wounds or branch stubs along the stem). A subsequent trial (limited to a single strain) with shorter pieces showed promise. Good basidiocarp development occurred in much less time with 10-cm-long stem sections, and without mould problems.

A complementary improvement to the method is to stand the short stem sections on end, raised slightly above the bench surface. Under these conditions the fungus fruits abundantly on the undersurface of the stem section. Optimal results in the limited trial with shorter stem sections occurred when the sections were also stood on end. In addition, a replicate of the DAOM 218218 strain which had not produced any fruiting because of mould was crosscut 1 cm back from the end face and stood on end. In less than 2 weeks it produced fruiting which exceeded any that this strain produced in the main experiment. In nature most fruiting of *C. purpureum* occurs on the underside of infected branches. This method is probably so successful

because it combines optimum conditions for fruiting: quick colonization of the wood, and an exposed wood surface on the underside of the infected host.

Basidiocarp storage. *Effect of temperature on spore production* - The total failure of the frozen-while-wet basidiocarps to produce spores was unexpected. In previous preliminary tests of shorter duration, basidiocarps stored in this manner produced some spores.

An equipment problem encountered during the experiment may have contributed to the failure of these basidiocarps to produce spores. The optimal temperature for *C. purpureum* spore production is 18°C (Spiers 1985). The growth chamber in which the basidiocarps were incubated was unable to maintain this temperature during the experiment. The minimum temperature reached was 21°C, and for much of the time the temperature in the growth chamber exceeded 25°C. Spiers (1985) found that basidiospore release decreased sharply at temperatures above 18°C, falling by 25°C to a level equal to that observed at -5°C.

Normally, basidiocarp enlargement is associated with a high level of spore production (Dye 1974; pers. observ.). A pale mauve edge develops on the enlarging basidiocarp. There was no such evidence of enlargement on the frozen-while-wet basidiocarps, and very little on the air-dried basidiocarps, even 24 hours after the experiment was completed. This is a further indication that the

incubation conditions were not optimal. It is noteworthy that in spite of the above-optimal temperature conditions the fungus was still very prolific over the 9 hours duration of the experiment, releasing up to 3.3 million spores per cm^2 of hymenium.

Freezing damage - Post-experiment microscopic examination of hymenial and sub-hymenial tissue from both the air-dried and frozen-while-wet basidiocarps showed that the basidia and hyphae of the air-dried basidiocarps had survived the 7 months period of freezing and subsequent thawing but the basidia of the basidiocarps which had been frozen while wet had been damaged.

Freezing damage to cells can occur in a variety of ways. As cooling proceeds to sub-zero temperatures, ice crystals form first in the extracellular spaces where the water has a lower concentration of solutes than inside the cell. If the cooling rate is relatively slow, or the plasma membrane is very permeable, the initially supercooled protoplasm equilibrates by losing water to the extracellular ice and does not freeze. If the cooling rate is too fast or the plasma membrane is not sufficiently permeable to water movement out of the cell, lethal intracellular freezing may occur (Mazur 1960).

It is unlikely that intracellular freezing caused the death of the frozen-while-wet hymenium. The fungal plasma membrane allows free movement of water in and out of the

cytoplasm (Rayner & Boddy 1988). In addition, the rate of cooling sustained by the basidiocarps when they were put in the freezer (at $-15^{\circ}C$) was at most $3^{\circ}C$/min (pers.observ.), much lower than the "critical cooling rate" of $10^{\circ}C$/min Mazur (1970) determined for yeast cells.

Nor is it likely that deleterious "solution effects" resulting from the concentration and precipitation of solutes associated with the removal of liquid water and its conversion to ice (Mazur 1970) caused the damage to the hymenium of the experimental material. *Chondrostereum purpureum* is well adapted to withstand dehydration, and presumably, the resultant increase in concentration of solutes. Spiers and Hopcroft (1988) observed numerous membrane-bound vacuoles formed in the cytoplasm of basidiospores, basidia, and subtending tissue of dehydrated basidiocarps. Within 2 hours of rehydration most of the vacuoles had disappeared, and within 4 hours the basidia were once again producing basidiospore initials.

Mechanical damage from extracellular ice crystals is the most probable cause of the deterioration of the hymenium of the frozen-while-wet basidiocarps. Ice crystals may have ruptured or damaged cell walls. In addition, the formation of minute ice crystals in the interstitial spaces in the highly porous cell wall (Rayner & Boddy 1988) during the freezing, and their subsequent growth during thawing (Mazur

1970), may account for the observed thickening of the hyphal cell walls.

Storage in glass vs. paper - Post-experiment microscopic examination of the hymenial surfaces of air-dried basidiocarps revealed no differences between those stored in glass sample jars and those stored in paper envelopes.

The difference in the level of spore release between the basidiocarps stored according to the two methods may simply be the result of a difference in the time required to fully rehydrate the cells. The basidia of the basidiocarps stored in paper envelopes would have been subjected to a steeper vapour pressure gradient in the freezer compartment than those in the small glass containers. Thus, these basidiocarps actually may have been drier than those stored in glass after 7 months in the freezer and therefore only needed more time to fully rehydrate and begin spore production.

Conversely, the extra drying of the basidiocarps stored in paper envelopes may have caused damage to some basidia, damage that was not detected by light microscopy.

Conclusions and recommendations

Basidiocarp production. *Chondrostereum purpureum* basidiocarps can be grown successfully in a greenhouse. Stem sections of birch approximately 10 cm long by 5 cm in diameter provide an adequate substrate for growth. Optimal

productivity can be attained by setting the inoculated stem sections on end, raised above the bench surface.

Basidiocarp storage. *Chondrostereum purpureum* basidiocarps can be stored frozen for several months (at least) and be restored to a normal sporulating state without damage, if properly stored. Before freezing, basidiocarps should be air-dried and placed in small sealed glass containers to limit further drying during storage. The optimal temperature for spore production is 18^{o}C. Incubation at a temperature below or above this optimum results in a diminished level of spore release.

Summary and Recommendations

Chondrostereum purpureum infects a wide range of hosts throughout the temperate zones of the world. Although its impact on fruit trees has been studied extensively, its pathogenicity to other species remains largely unexplored.

The survey of the association of *C. purpureum* with the dieback and decline of white birch showed that *C. purpureum* was consistently associated with symptomatic trees. The observed progression of dieback and decline was identical to that observed by Cunningham (1922) in diseased trees in New Zealand.

The pathogenicity of *C. purpureum* to birch seedlings was demonstrated according to Koch's postulates. The fungus caused cankers to form and internal infections in the xylem, as observed by Wall (1986) in yellow birch. In addition, one seedling was killed and progressive dieback occurred in another. The virulence of the pathogen was demonstrated through its ability to spread rapidly and overcome compartmentalization in at least two vigorously growing 3-year-old seedlings.

A key factor in the pathogenicity of *C. purpureum* is the mycotoxin production. The total number of toxins produced by the fungus remains a mystery, but apparently there are at least two (Brooks & Brenchley 1929). In this study, identification of a possibly toxic compound which formed needle-like crystals on heavy spore casts was not accomplished, but its properties were studied. The

compound was a sesquiterpene similar to, and possibly identical to, (+)-torreyol, recently reported to have produced crystals in cultures of *Stereum hirsutum* (Ainsworth *et al.* 1990).

The results of a study of the sexuality of *C. purpureum* agreed substantially with those of earlier reports (Robak 1936, 1942). *Chondrostereum purpureum* is heterothallic with tetrapolar sexual differentiation. The study went beyond that of earlier work and revealed unilateral compatibility between monokaryons as well as unequal abundance of clamps and fruiting in compatible pairings. These observations raise questions regarding the mechanisms controlling nuclear migration, fruiting competence, and nuclear dominance in dikaryons.

Chondrostereum purpureum basidiocarps can be grown on pieces of birch inoculated with infected patches of bark. This method would prove very useful when studying strains which are currently available only in culture. Within 2 to 3 months, the researcher can have representative basidiocarps for the strains under study. Likewise, storage of basidiocarps in a viable condition is important for studies requiring spores, such as studies of sexuality and pathogenicity. Air-dried basidiocarps can be stored frozen in glass containers.

The mycotoxin which produces the silver leaf symptom has been the focus of virtually all the toxicologic research

directed at *C. purpureum*. Future research should concentrate on determining the number, properties, and effects of the other mycotoxins produced by *C. purpureum*. Perhaps the striking effect of the silvering toxin on susceptible species has masked the presence and impacts of other toxins, such as those which may cause stomatal closure and contribute to starvation (Spiers *et al.* 1987) and predisposition to attacks by other organisms (e.g. bronze birch borer).

If white birch is to remain a component of the urban forest, homeowners and municipal employees must be educated in proper methods of landscaping and tree maintenance. These attractive but vulnerable trees must be protected from activities that cause wounds and provide infection courts.

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Appendices

Appendix I

Water demands of inoculated and non-inoculated seedlings under water stress

TC2 -	Tree # 11	13	23	22	29	30	33	43	47	53	55	56	73
days between watering	21	13	22	30	18	15	12	15	19	19	17	18	21
	12	13	7	9	11	10	10	6	11	11	11	10	11
		8		9	9	7	4	11	8	8	9	9	
		7			7	6	9						
						7	8						
times water was added	2	4	2	2	3	4	5	5	3	3	3	3	3

average number of wilts = 3.2
average # days between watering = 11.9

TC4 -	Tree # 1	6	21	37	40	49	51	52	59	61	69	71	74
	11	12	13	13	12	12	12	12	16	14	18	12	16
	4	9	9	12	9	9	7	9	9	10	8	9	10
	10	8	7	7	8	8	7	6	8	9	8	8	7
	7	7	7	6	3	7	6	5	5	8	8	8	8
	6		6		10	6	5	4					
							6						
times water was added	5	4	5	4	5	5	6	5	4	4	4	4	4

average number of wilts = 4.5
average # days between watering = 8.7

Test of hypotheses (1) wilts

Test concerning two means, μ_1 and μ_2, with σ_1^2 and σ_2^2 unknown but assumed equal.

Null Hypothesis H_0 : $\mu_1 = \mu_2$; i.e., that the number of wilts experienced by the non-inoculated and inoculated seedlings over 42 days was equal.

Alternative Hypothesis H_1 : $\mu_1 > \mu_2$; ; i.e. that the number of wilts experienced by the non-inoculated seedlings is significantly greater ($\alpha = 0.001$, $t > 3.745$) than that experienced by the inoculated seedlings.

Test Criteria: reject H_0 if $t > 3.745$, where t is calculated as described below.

Test Statistic (Distribution) -

$$t_{(n_1+n_2-2)} = \frac{\bar{x}_1 - \bar{x}_2}{s_p\sqrt{(1/n_1 + 1/n_2)}} \qquad \text{(Student-t)}$$

$$\text{and } s_p^2 = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2}$$

Calculations

Statistics for <u>wilts by non-inoculated seedlings</u>:

Number of non-inoculated seedlings (n_1) = 13

Average number of wilts = 4.538

Sample variance (s_1^2) = 0.4359

Statistics for <u>wilts by inoculated seedlings</u>:

Number of inoculated seedlings (n_2) = 13

Average number of wilts = 3.23

Sample variance (s_2^2) = 1.0256

Test of variances:

$F_{(n_2-1),(n_1-1)} = s_2^2/s_1^2$

$F_{12,12}$ critical value ($F_{.05}$) = 2.69

$s_2^2/s_1^2 = 1.0256/0.4359$

$= 2.35$, therefore, $\sigma_1^2 = \sigma_2^2$

Pooled variance:

$$s_p^2 = \frac{(13-1)0.4359 + (13-1)1.0256}{13 + 13 - 2}$$

$$= 0.73075$$

$$s_p = \sqrt{0.73075}$$

$$= 0.854839$$

Calculation of Student-t:

$$t_{24} = \frac{4.538 - 3.230}{0.8548\sqrt{(1/13+1/13)}}$$

$$= \frac{1.308}{0.33529}$$

$$= 3.90$$

The experimental t value, 3.90, exceeds the t_{24} critical value 3.745 ($\alpha = 0.001$), therefore reject the null hypothesis that $\mu_1 = \mu_2$, and conclude that the number of wilts experienced by the non-inoculated seedlings is significantly greater ($\alpha < 0.001$) than that by the inoculated seedlings.

Test of hypotheses (2) days between watering

Test concerning two means, μ_1 and μ_2, with σ_1^2 and σ_2^2 unknown but assumed not equal.

Null Hypothesis H_0 : $\mu_1 = \mu_2$; i.e., that the number of days between watering of the inoculated seedlings and of the non-inoculated seedlings over 42 days was equal.

Alternative Hypothesis H_1 : $\mu_1 > \mu_2$; ; i.e. that the number days between watering of the inoculated seedlings was significantly greater ($\alpha = 0.01$, $t > 2.66$) than that experienced by the non-inoculated seedlings.

Test Criteria: reject H_0 if $t > 2.66$, where t is calculated as described below.

Test Statistic (Distribution) -

$$t_\delta = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{((s_1^2/n_1) + (s_2^2/n_2))}}$$

estimate of Student-t; δ is an adjusted value allowing for estimation.

Calculations

Statistics for number of days between watering of the inoculated seedlings:

Number of watering of inoculated seedlings (n_1) = 42

Average number of days between watering = 11.857

Sample variance (s_1^2) = 29.198

Statistics for number of days between watering of the non-inoculated seedlings:

Number of watering of non-inoculated seedlings (n_2) = 59

Average number of days between watering = 8.745

Sample variance (s_2^2) = 9.434

Test of variances:

$F_{(n_1-1),(n_2-1)} = s_1^2/s_2^2$

$F_{41,58}$ critical value ($F_{.05}$) = 1.59

$s_1^2/s_2^2 = 29.198/9.434$

$= 3.09$, therefore, $\sigma_1^2 > \sigma_2^2$

Calculation of estimate of t:

$$t_{60} = \frac{11.857 - 8.745}{\sqrt{(29.1981/42+9.434/59)}}$$

$$= \frac{3.112}{0.9247}$$

$$= 3.36$$

The experimental t value, 3.36, exceeds the t_{60} critical value 2.66 ($\alpha = 0.01$), therefore reject the null hypothesis that $\mu_1 = \mu_2$, and conclude that the number of days between watering for the inoculated seedlings is significantly greater ($\alpha < 0.01$) than that for the non-inoculated seedlings.

Appendix II

ANOVA of height increment

Height increment since inoculation:

REP #	TC1	TC2	TC3	TC4
1	27.0	33.2	36.0	17.7
2	19.2	18.5	40.8	15.0
3	31.3	0.0	35.2	16.5
4	20.3	0.7	26.3	13.7
5	31.2	17.9	33.6	10.7
6	37.2	24.9	19.1	19.3
7	22.0	21.3	39.1	28.2
8	41.1	5.4	23.2	27.7
9	18.0	10.7	35.1	23.2
10	23.1	21.1	21.6	18.4
11	39.8	13.4	47.2	26.0
12	36.5	14.6	25.2	15.4
13	48.2	18.2	41.3	12.9
SUM	394.9	199.9	423.7	244.7
AVG	30.4	15.4	32.6	18.8
n =	52			
TOTAL =		1263.2		
G. MEAN=		24.3		

	inoculated	non-inoculated	totals
watered	394.9	423.7	818.6
stressed	199.9	244.7	444.6
totals	594.8	668.4	1263.2

CF = T^2/n = 30686.0

SUM x^2 = 36992.4

SS = 6306.4

SS(treatments) = 2799.0

SS(pathogen) = 104.2 1 df

SS(water) = 2689.9 1 df

SS(path+water) = 4.9 1 df

SSE = 3507.3 48 df

ANOVA

Source	df	SS	MS	F	F 0.05
pathogen	1	104.2	104.2	1.426	4.04
water	1	2689.9	2689.9	36.813	4.04
pXw	1	4.9	4.9	0.067	4.04
error	48	3507.3	73.1		
totals	51	6306.4			

Appendix III

Instrumentation tests details

XRD values for crystals:

intensity ranking Line #	S_1 mm	S uncorr $(S_1-\theta_1)$	S corr	$S/2(\theta^{o})$	sinθ	2sinθ	d
4	147.10	12.37	12.39	6.19	0.1079	0.216	7.13
8	156.15	21.42	21.46	10.73	0.1862	0.372	4.13
7	154.20	19.47	19.51	9.75	0.1694	0.339	4.54
5	150.95	16.22	16.25	8.12	0.1413	0.283	5.44
6	153.20	18.47	18.50	9.25	0.1608	0.322	4.78
9	161.00	26.27	26.32	13.16	0.2276	0.455	3.38
1	140.85	6.12	6.13	3.06	0.0535	0.107	14.38
3	142.90	8.17	8.19	4.09	0.0714	0.143	10.78
2	142.00	7.27	7.28	3.64	0.0635	0.127	12.11

θ_1 = (113.3+156.16)/2 = 134.725 mm
θ_2 = 314.4 - θ_1 = 179.675
correction factor = 180/179.675
= 1.0018
S/2 = Bragg angle in degrees since 2 mm = 1^{o} Bragg
d = lambda/2*sinθ

Analysis of instrumentation results:

On the basis of preliminary elemental analysis, biogenetic considerations, and mass spectroscopy (M^{+} =222), we suggest that the compound has a molecular formula of $C_{15}H_{26}O$. The mass spectrum shows an intense peak at m/e = 204 which is probably due to the loss of water. The IR spectrum (carried out on a single crystal) showed absorbance at 3300 cm^{-1} suggesting the presence of a

hydroxyl group. There was no absorbance in the carbonyl region of the spectrum.

Ayer *et al.* (1981) have isolated a number of compounds from *C. purpureum*. They have established the structure of these compounds and they all contain a four membered carbocyclic ring. The presence of the cyclobutane ring gives rise to a very intense M^{+} -28 peak in the mass spectrum. The M^{+} -28 peak was not apparent in the mass spectrum of the compound that we isolated.

The C^{13} spectrum has 15 or 16 distinct peaks (the exact number depends on what one considers background). All but two of these peaks have a chemical shift of less than 45 ppm, consistent only with a saturated hydrocarbon type structure. The peak at approximately 75 ppm (if indeed it is real) could be due to a carbon with an attached oxygen. The two peaks at 125 ppm and 134 ppm could be due to double bond carbons, however the peak at 134 ppm might be due to background noise.

The H^{1} NMR spectrum also indicates a compound which is mainly hydrocarbon in nature. With the exception of a peak at 5.5 ppm, all peaks are between 2.0 ppm and 0.8 ppm. The four sharp peaks in the vicinity of 0.8 ppm suggest four high field methyl groups. The small peak at 5.5 ppm could be due to a proton on a carbon bearing an oxygen atom. (Interpretation provided by Dr.D. Orr, Lakehead University.)

Significant peaks in the mass spectrum of the *C. purpureum* compound (M^+ 222):

Peak	Relative Intensity[a]
204	60%
187	38%
161	100%
159	44%
119	44%
105	66%

[a] intensity of peak relative to base peak, m/e = 161.

Appendix IV

A list of mating types and pairings

Mating types

Mating Type	Single-spore Isolate No.
A_1B1	1
A_1B_2	4, 5, 7, 10, 12
A_2B_2	2, 3, 6, 8, 9
A_2B_1	11

Pairings

Positive:

1. A_1B_1 X A_2B_2 = 1,2 1,3 1,6 1,8 1,9

6. A_1B_2 X A_2B_1 = 4,11 5,11 7,11 10,11 12,11

Negative:

2. A_1B_1 X A_1B_2 = 1,4 1,5 1,7 1,10 1,12

3. A_1B_1 X A_2B_1 = 1,11

4. A_2B_2 X A_1B_2 = 2,4 2,5 2,7 2,10 2,12 3,4 3,5 3,7 3,10 3,12 6,4 6,5 6,7 6,10 6,12 8,4 8,5 8,7 8,10 8,12 9,4 9,5 9,7 9,10 9,12

5. A_2B_2 X A_2B_1 = 2,11 3,11 6,11 8,11 9,11

7. A_2B_2 X A_2B_2 = 2,3 2,6 2,8 2,9 3,6 3,8 3,9 6,8 6,9 8,9

8. A_1B_2 X A_1B_2 = 4,5 4,7 4,10 4,12 5,7 5,10 5,12 7,10 7,12 10,12

9. A_1B1 X A_1B_1 = 1,1

10. A_2B_1 X A_2B_1 = 11,11

Appendix V

Calculations of spore production

Basidiocarps stored dry in glass:

	DG1	DG2	DG3	DG4	DG5
Hemacytometer readings, i.e. no. of spores in $0.1mm^3$ water.	13 12 11 21 15	11 22 9 13 17	11 6 10 7 13	25 11 16 12 11	14 18 14 16 17
avg/$0.1mm^3$	14.4	14.4	9.4	15	15.8
ml of water in petri dish	27	27	28	29	29
total spore production	3888000	3888000	2632000	4350000	4582000
FB area cm^2	1.3	1.4	0.9	1.3	1.0
spore prod./ cm^2 FB	2990769	2777143	2924444	3346154	4582000

DG avg 3324102 spores/cm^2 hymenium
DG std 733620
DG var 5.38E+11

Basidiocarps stored dry in paper envelopes:

	DP1	DP2	DP3	DP4	DP5
Hemacytometer readings, i.e. no. of spores in $0.1mm^3$ water.	6 18 6 7 7	14 14 15 7 6	12 3 8 3 6	8 4 10 7 8	21 18 9 16 20
avg/$0.1mm^3$	8.8	11.2	6.4	7.4	16.8

ml of water in petri dish	27	29	27	28	28
total spore production	2376000	3248000	1728000	2072000	4704000
FB area cm^2	1.4	1.4	1.1	1.2	2.2
spore prod./ cm^2 FB	1697143	2320000	1570909	1726667	2138182

DP avg 1890580 spores/cm^2 hymenium
DP std 321007
DP var 1.0E+11

Test of variances: DGvar/DPvar = 5.222907
F 4,4 (p=0.05) = 6.39

Test of hypotheses

Test concerning two means, μ_1 and μ_2, with σ_1^2 and σ_2^2 unknown but assumed equal.

Null Hypothesis H_0 : $\mu_1 = \mu_2$; i.e., that the spore production from the dried basidiocarps stored in glass containers equals that of the dried basidiocarps stored in paper envelopes.

Alternative Hypothesis H_1 : $\mu_1 > \mu_2$; ; i.e. that the spore production from the dried basidiocarps stored in glass containers is significantly greater ($\alpha = 0.05$, $t > 1.860$) from that of the dried basidiocarps stored in paper envelopes.

Test Criteria: reject H_0 if $t > 1.860$, where t is calculated as described below.

Test Statistic (Distribution) -

$$t_{(n_1+n_2-2)} = \frac{\bar{x}_1 - \bar{x}_2}{s_p\sqrt{(1/n_1 + 1/n_2)}} \qquad \text{(Student-t)}$$

$$\text{and } s_p^2 = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2}$$

Calculations

Statistics for basidiocarps stored in glass:

Number of basidiocarps (n_1) = 5

Average spore production per cm² ($\bar{x}_1$) = 3 324 102

Sample variance (s_1^2) = 5.38E+11

Statistics for basidiocarps stored in paper envelopes:

Number of basidiocarps (n_2) = 5

Average spore production per cm² ($\bar{x}_2$) = 1 890 580

Sample variance (s_2^2) = 1.03E+11

Test of variances:

$F_{(n_1-1),(n_2-1)} = s_1^2/s_2^2$

$F_{4,4}$ critical value ($F_{.05}$) = 6.39

s_1^2/s_2^2 = (5.38E+11)/(1.03E+11)

= 5.22, therefore, $\sigma_1^2 = \sigma_2^2$

Pooled variance:

$$s_p^2 = \frac{(5-1)5.38E+11 + (5-1)1.03E+11}{5 + 5 - 2}$$

= 3.206219E+11

$s_p = \sqrt{3.206219E+11}$

= 566235

Calculation of Student-t:

$$t_8 = \frac{3324102 - 1890580}{566235\sqrt{(1/5+1/5)}}$$

$$= \frac{1433522}{358118}$$

= 4.003

The experimental t value, 4.003, exceeds the t_8 critical value 1.860 (α = 0.005), and also 3.833 (α = 0.0025), therefore reject the null hypothesis that $\mu_1 = \mu_2$, and conclude that the spore production from the air-dried basidiocarps stored in glass containers is significantly greater ($\alpha < 0.0025$) than that from the air-dried basidiocarps stored in paper envelopes.