

**Assessment of *Neonectria neomacrospora* as a Potential Biological
Control Agent for Hemlock Dwarf Mistletoe**

By

Lea Mae Rietman

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Abstract

The purpose of this study was to assess the potential of *Neonectria neomacrospora* as an inundative biological control agent for hemlock dwarf mistletoe (*Arceuthobium tsugense*). Based on the growth characteristics of six isolates of *N. neomacrospora*, isolate PFC 2559 was selected as the inoculum source. A field trial was conducted on Vancouver Island to determine the requirement of wounding for *N. neomacrospora* infection and to measure the impact of *N. neomacrospora* infection on *A. tsugense* vigour. Conidia were mass produced and suspended in "Stabileze" formulation, which was sprayed onto entire hemlock dwarf mistletoe infections (swellings and shoots). Treatments involved unwounded *A. tsugense* swellings, cut *A. tsugense* aerial shoots, and wounded *A. tsugense* swellings. The number of healthy and diseased *A. tsugense* aerial shoots was recorded at 2 weeks and 1, 2, 3.5, 5, 6, and 9 months after treatment. At 10 months, a sub-portion of the treated *A. tsugense* swellings was destructively sampled and an attempt to re-isolate *N. neomacrospora* was done. The greatest amount of re-isolation of *N. neomacrospora* occurred for the wounded, inoculated treatment (73%), followed by the cut shoots, inoculated treatment (60%), and the unwounded, inoculated treatment (55%); (35%, 20%, and 21% for controls, respectively). Based on the extent of bark necrosis, the occurrence of sporodochia, and re-isolation of *N. neomacrospora*, the wounded, inoculated treatment had the greatest impact on *N. neomacrospora* infection when compared to the unwounded, inoculated treatment and the cut shoots, inoculated treatment. There was no evidence that *N. neomacrospora* could infect wounded *A. tsugense* shoots. Once infection by *N. neomacrospora* was established, a 38% decline in the number of healthy *A. tsugense* aerial shoots was observed at 9 months. Applying inoculum to unwounded *A. tsugense* swellings is predicted to reduce the number of *A. tsugense* shoots by 14% in the field at 9 months. This is a conservative estimate. Due to the extensive bark necrosis and girdling of the *A. tsugense* swelling, infection by *N. neomacrospora* is expected to have a greater long-term impact on *A. tsugense* vigour than that observed in the present field trial.

Table of Contents

Abstract.....	ii
Table of contents.....	iii
List of tables.....	vii
List of figures.....	ix
Acknowledgments.....	xii

Chapter 1 **Literature Review**

1.10 Overview of the role of forest pathogens.....	1
1.2.0 Dwarf mistletoes.....	1
1.2.1 Background on dwarf mistletoes.....	1
1.2.2 Symptoms and signs of dwarf mistletoes.....	2
1.2.3 Life cycle of dwarf mistletoes.....	3
1.2.4 Biology of dwarf mistletoes.....	4
1.2.5 Distribution of dwarf mistletoes.....	7
1.2.6 Hosts of dwarf mistletoes.....	7
1.2.7 Impact of dwarf mistletoe in forestry.....	8
1.2.8 Spread and intensification of dwarf mistletoes.....	9
1.3.0 Management strategies for dwarf mistletoes.....	10
1.3.1 Traditional control of dwarf mistletoes.....	12
1.3.2 Chemical control of dwarf mistletoes.....	14
1.3.3 Genetic control of dwarf mistletoes.....	15
1.3.4 Biological control of dwarf mistletoes.....	17

1.3.4.1	Hyperparasites of dwarf mistletoe shoots.....	19
1.3.4.2	Hyperparasites of dwarf mistletoe swellings.....	22
1.4.0	Introduction to <i>Arceuthobium tsugense</i>	23
1.4.1	Symptoms and signs of <i>A. tsugense</i>	23
1.4.2	Biology of <i>A. tsugense</i>	23
1.4.3	Distribution of <i>A. tsugense</i>	25
1.4.4	Taxonomy and hosts of <i>A. tsugense</i>	26
1.4.5	Spread and intensification of <i>A. tsugense</i>	27
1.4.6	Impact of <i>A. tsugense</i> in forestry.....	28
1.4.7	Control of <i>A. tsugense</i>	30
1.5.0	Introduction to <i>N. neomacrospora</i>	30
1.5.1	Taxonomy and hosts of <i>N. neomacrospora</i>	31
1.5.2	Symptoms and signs of <i>N. neomacrospora</i>	33
1.5.3	Biology of <i>N. neomacrospora</i>	36
1.5.4	A potential biological control agent of <i>Arcuethobium tsugense</i>	37
1.6.0	Research objectives.....	38

Chapter 2

Selection of an isolate of *Neonectria neomacrospora*

2.1.0	Introduction.....	39
2.2.0	Materials and methods.....	39
2.2.1	Collection of <i>Neonectria neomacrospora</i> isolates.....	40
2.2.2	Selection and screening of the <i>N. neomacrospora</i> isolates.....	41
2.2.3	Linear colony growth at varying temperatures.....	41

2.2.4 Percentage and rate of spore germination at varying temperatures.....	42
2.2.5: Mass production of inoculum on different solid -based media.....	44
2.2.6 Inoculation of <i>A. tsugense</i> in a small field trial.	44
2.3.0 Results.....	48
2.3.1 Selection and screening of the <i>N. neomacrospora</i> isolates	48
2.3.1.1 Linear colony growth at varying temperatures.....	48
2.3.1.2 Percentage and rate of spore germination at varying temperatures	50
2.3.1.3 Mass production of inoculum on different solid-based media	52
2.3.2 Inoculation of <i>A. tsugense</i> in the small field trial	54
2.4.0 Discussion	56
2.4.1 Growth characteristics of <i>N. neomacrospora</i>	56
2.4.2 Small field trial.....	57

Chapter 3
Inoculation of *Arceuthobium tsugense* in a large field trial

3.1.0 Introduction.....	60
3.2.0 Materials and methods.....	61
3.2.1 Stabileze formulation.....	61
3.2.2 Site description.....	62
3.2.3 Experimental design.....	63
3.2.4 Pre-treatment assessment.....	65
3.2.5 Treatment application dates.....	65

3.2.6 Treatment assessment.....	65
3.2.7 Sampling at 10 months.....	65
3.2.8 Statistical analysis.....	67
3.2.8.1 Statistical analysis of the impact of wounding on infection.....	67
3.2.8.2 Statistical analysis of infection on <i>A. tsugense</i> vigour.....	68
3.3.0 Results	69
3.3.1 Stabileze formulation	69
3.3.2 Symptoms and signs of <i>N. neomacrospora</i> in the field.....	70
3.3.3 Lost experimental units 10 months following inoculation.....	72
3.3.4 Importance of wounding for establishment of <i>N. neomacrospora</i>	73
3.3.4.1 Extent of bark necrosis.....	74
3.3.4.2 Proportion of <i>A. tsugense</i> with sporodochia.....	75
3.3.4.3 Re-isolation of <i>N. neomacrospora</i> from <i>A. tsugense</i> swellings.....	78
3.3.5 Impact of <i>N. neomacrospora</i> on <i>A. tsugense</i> vigour.....	81
3.3.5.1 Number of healthy <i>A. tsugense</i> shoots for unconfirmed and confirmed groups.....	79
3.3.5.2 Proportion of <i>A. tsugense</i> infections with zero shoots for unconfirmed and confirmed groups.....	83
3.3.5.3 Extent of bark necrosis and girdling of <i>A. tsugense</i> swellings for unconfirmed and confirmed groups.....	83
3.4.0 Discussion	85
3.4.1 Stabileze formulation	85
3.4.2 Importance of wounding for establishment of <i>N. neomacrospora</i>	86

3.4.3	Effect of <i>N. neomacrospora</i> infection on <i>A. tsugense</i> vigour.....	89
3.4.4	Measuring the impact of treatment A on <i>A. tsugense</i> shoot vigour.....	90
3.5.0	Closing Remarks.....	92
3.6.0	Future Research.....	93
	Literature Cited.....	94
	Appendix I-HOBO data for small field trial- temperature.....	105
	Appendix II-HOBO data for small field trial- relative humidity.....	106
	Appendix III-HOBO data for large field trial-temperature	107
	Appendix IV-HOBO data for large field trial-relative humidity.....	108

List of Tables

Table 1.1: Morphology and host characteristics of <i>Nectria fuckeliana</i> var <i>fuckeliana</i> and <i>Neonectria neomacrospora</i> (modified from Funk 1981; Booth 1979a, 1979b)..	32
Table 2.1. The origin of the six isolates of <i>N. neomacrospora</i> selected for screening.	41
Table 2.2. Treatment description and number of replicates for each treatment used in the small field trial.....	46
Table 2.3. Sporodochia and successful re-isolation of <i>N. neomacrospora</i> (N. n.) for each treatment from the small field trial. The proportion of <i>A. tsugense</i> swellings colonized by <i>N. neomacrospora</i> was based on the occurrence of sporodochia only.....	55
Table 3.1. Treatment description and number of replicates used in the field trial designed to assess <i>N. neomacrospora</i> as a biological control agent for <i>A. tsugense</i> .	64
Table 3.2. The number of experimental units collected from the trial 10 months after treatment. Note that the total number of experimental units ("Total") takes into account <i>A. tsugense</i> infections that were lost during the trial (Table 3.4).	66
Table 3.3. Break down of the proportion of <i>A. tsugense</i> swellings with confirmed and unconfirmed <i>N. neomacrospora</i> infection for each treatment.	69
Table 3.4. The number of <i>A. tsugense</i> infections prior to treatment and the number of <i>A. tsugense</i> infections remaining at the termination of the trial.....	72
Table 3.5. Number of destructively sampled <i>A. tsugense</i> swellings at 10 months with and without sporodochia and successful re-isolation of <i>N. neomacrospora</i>	79

List of Figures

- Figure 1.1** Typical signs and symptoms of *Neonectria neomacrospora*.
A. Perithecia from the sexual stage of *N. neomacrospora*, B. Sporodochia from the asexual stage of *N. neomacrospora*, C. Macroconidia from the asexual stage of *N. neomacrospora*, D. Mycelium of the asexual stage of *N. neomacrospora* grown on potato dextrose agar for 21 days, E. Resinosis and cankering of the *A. tsugense* swelling, F. Destructively sampled *A. tsugense* swelling with top layer of bark removed to show bark necrosis, G. Cross section of an *A. tsugense* swelling showing bark necrosis, H. Diseased and dead *A. tsugense* shoots. Pictures A and E courtesy Dr. Simon Shamoun, Pacific Forestry Centre, Victoria, BC (2003)..... 33-34
- Figure 2.1.** A sample graph of the percentage of germinated spores plotted against time for PFC 2546 at 20°C. In this case, $r^2 = 0.95$ 43
- Figure 2.2.** Linear colony growth (mm/year) of six isolates of *N. neomacrospora* at temperatures ranging from 4 to 30°C. Bars measure Standardn error of the mean.....48
- Figure 2.3.** Colony radius (mm) across time for six isolates of *N. Neomacrospora* at 20°C. Bars measure standard error of the means.....49
- Figure 2.4.** Percent conidial germination for six isolates of *N. neomacrospora* 24 hours after inoculation at temperatures ranging from 4°C to 35°C.....50
- Figure 2.5.** Number of hours for 50% spore germination for six isolates of *N. neomacrospora* at temperatures ranging from 4°C to 30°C.....51
- Figure 2.6.** Mean number of spores produced by six isolates of *N. neomacrospora* averaged across four different media types (brown rice, millet, wheat bran and slow oats) at room temperature (22°C). Bars measure standard error of the mean52
- Figure 2.7.** Mean number of spores produced on four media types averaged across six isolates of *N. neomacrospora* at room temperature (22°C). Bars measure standard error of the mean.53
- Figure 2.8.** Mean number of healthy *A. tsugense* shoots observed at 1 and 12 months after treatment. Bars measure standard error of the mean.54

Figure 2.9. Symptoms of <i>A. tsugense</i> infected with a mycelial plug of <i>N. neomacrospora</i> into a wound at 1 month (A) and 9 months (B). The arrow in (A) points to the site of inoculation. Note the diseased, dead <i>A. tsugense</i> aerial shoots and girdling of the swelling at 9 months (B).....	58
Figure 3.1. A treated <i>A. tsugense</i> swelling at 2 weeks (A) and 9 months (B) after treatment. Treatment involved wounding the <i>A. tsugense</i> swelling and applying inoculum (treatment C). Infection by <i>N. neomacrospora</i> was confirmed.	71
Figure 3.2. A treated <i>A. tsugense</i> swelling at 2 weeks (A) and 9 months (B) after treatment. Treatment involved applying inoculum to unwounded <i>A. tsugense</i> swellings (treatment A). Infection by <i>N. neomacrospora</i> was not confirmed.	71
Figure 3.3. Mean extent of bark necrosis for <i>A. tsugense</i> swellings collected 10 months after treatment. Bars measure standard error of the mean.....	74
Figure 3.4. Proportion of <i>A. tsugense</i> swellings collected 10 months after treatment bearing confirmed sporodochia.	75
Figure 3.5. Cumulative proportion of <i>A. tsugense</i> swellings bearing sporodochia 3.5, 6 and 9 months after treatment.	76
Figure 3.6. The non-cumulative proportion of <i>A. tsugense</i> swellings bearing sporodochia. All treatments are grouped together for this data.	77
Figure 3.7. Proportion of <i>A. tsugense</i> swellings from which <i>N. neomacrospora</i> was successfully re-isolated 10 months after treatment.	78
Figure 3.8. Linear relationship of the extent of bark necrosis and the proportion of <i>A. tsugense</i> swellings from which <i>N. neomacrospora</i> was successfully re-isolated. Regression analysis of $Y=Y_0 + aX$ found $r^2 = 0.93$	80
Figure 3.9. Mean number of healthy <i>A. tsugense</i> shoots 9 months after treatment for unconfirmed and confirmed groups. Cut shoot treatments were removed from analysis. Bars measure standard error of the mean.	81

Figure 3.10. Mean number of healthy *A. tsugense* shoots observed for unconfirmed and confirmed *N. neomacrospora* infected groups. Bars measure standard error of the mean.82

Figure 3.11. Mean proportion of *A. tsugense* infections with no shoots 10 months after treatment for unconfirmed and confirmed groups.83

Figure 2.20. Proportion of girdled *A. tsugense* swellings 10 months after treatment for unconfirmed and confirmed groups.....84

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Chapter 1: Literature Review

1.1.0 Overview of the role of forest pathogens

“Disease” can be defined as the deleterious effects resulting from injurious biotic agents other than insect damage or animal wounding (Callan 2001). A biotic agent that incites disease in a host is termed a “pathogen” (Castello, Leopold, and Smallidge 1995). Both pathogens and abiotic disturbances recycle essential elements and influence forest development and landscape, often selecting for less vigorous or genetically susceptible individuals. Pathogens are unique in that they require the presence of a susceptible host and favourable environmental conditions (Callan 2001).

Pathogens are major agents of forest diversity (van der Kamp 1991). The primary route in which pathogens impact forest succession is through tree mortality. Pathogens may impact (and be impacted by) plant species distributions, forest structure and composition, succession, biodiversity, and landscape patterns (Castello, Leopold, and Smallidge 1995).

Because forest pathogens can alter forest species composition and reduce host vigour, most forest pathogens are considered undesirable in the forest industry. Dwarf mistletoes, a genus of plant parasites that attack conifers, are a good example of problematic forest pathogens. Dwarf mistletoes cause extensive timber losses through direct and indirect mortality and growth reduction. They are especially damaging in parts of the Northern Hemisphere, particularly in western North America. In the USDA Forest Service survey from 1979-1983, the area of US commercial forest affected by dwarf mistletoe reached 22.2 millions of acres, surpassing root pathogens, fusiform rust, southern pine beetle, western spruce budworm and gypsy moth (USDA Forest Service 1988). The impact of dwarf mistletoes in forestry is significant and currently acceptable control methods are limited.

1.2.0 Dwarf mistletoe

1.2.1 Background on dwarf mistletoe

Dwarf mistletoes (*Arceuthobium*) belong to the family Viscaceae and are obligate, perennial plant parasites of Pinaceae and Cupressaceae in the Old and New Worlds.

Dwarf mistletoes rely on their host for support, mineral nutrients and water, some carbon compounds, and possibly other growth factors. The group consists of 46 recognized taxa, which comprise of 42 species, 4 with 2 subspecies each, and 1 with 2 *formae speciales* (Hawksworth and Wiens 1996).

Note: The term “infection” can be defined as the process of a pathogen establishing itself on a host and obtaining food (Agrios 1997) or as the pathological state resulting from having been infected (R. J. Copeman, University of British Columbia, Vancouver, BC, pers. comm.). In this document, both definitions were used. For example, infection may refer to the process of dwarf mistletoe parasitizing its host, or it may be used to refer to the entire dwarf mistletoe plant and parasitized host region.

1.2.2 Symptoms and signs of dwarf mistletoes

In most cases, the first symptom associated with infection by dwarf mistletoe is swelling of the host tissue. From the swelling arise dwarf mistletoe buds that develop into shoots. Dwarf mistletoe shoots are perennial and the fruit they bear is the sole means of reproduction. After a shoot is broken or falls off, a unique structure called a basal cup remains attached to the host (Hawksworth and Wiens 1996).

Older dwarf mistletoe infections often cause a phenomenon known as witches' broom, which consists of dense, profusely branched masses of host branches at the site of infection (Baranyay, Hawksworth, and Smith 1971). Witches' brooms can either be systemic (isophasic) or non-systemic (anisophasic). Typically, it is the parasite species rather than the host that determines the type of witches' broom formed. A few species of dwarf mistletoe, such as *Arceuthobium aureum* subsp. *aureum* and *A. globosum* subsp. *globosum*, do not induce witches' broom (Hawksworth and Wiens 1996).

Host vigour and wood quality may also be reduced by dwarf mistletoe infection. Heavily dwarf mistletoe infected trees have been shown to have shorter needles and needle bearing stem length, reduced leaf surface and number of needles, and chlorosis of the needles (Korstian and Long 1922). Wood quality of infected dwarf mistletoe trees is reduced by the production of large knots, development of abnormal grain, and reduction in strength (Anonymous 1985).

1.2.3 Life cycle of dwarf mistletoes

Dwarf mistletoes are dioecious seed plants. Depending on the species, the life cycle of mistletoe usually takes 5 or more years to complete (Hawksworth and Wiens 1996). The fruit contains a single seed and embryo. The seed is forcibly ejected and dispersed in late summer and early fall using a hydrostatically controlled explosive mechanism (Hinds, Hawksworth, and McGinnies 1963). Seeds can be discharged at velocities averaging 24 m per second (Hinds and Hawksworth 1965). Animal vectors may also play a role in dwarf mistletoe seed dispersal by disseminating seeds stuck to their feathers or fur. Some birds, such as ruffed grouse, blue grouse, and black-capped chickadee, have been reported to eat dwarf mistletoe seeds (Hawksworth 1975). Squirrels have also been known to feed on dwarf mistletoe infected bark (Baranyay, 1968, Hawksworth 1975). Although dwarf mistletoe seeds cannot survive through the digestive tract, feeding provides an opportunity for seeds to stick to the feathers or fur of the vector, which are removed later by grooming (Hudler, Oshima, and Hawksworth 1979; as cited in Hawksworth and Wiens 1996).

Dwarf mistletoe seeds adhere to their landing surface by means of a sticky, viscous coating (viscin) that surrounds the seed (Roth 1959). Roth (1959) observed that seeds were more prone to stick to needles rather than branch parts that were bare of needles, as the seeds tended to bounce off the more solid branch parts. Hawksworth (1965) noted that of those *Arceuthobium americanum* and *A. vaginatum* seeds that landed on a host, 90% and 94% of the seeds landed on the host needles, respectively.

Once wet with rain, the hygroscopic viscin becomes well lubricated and allows the seed to slide to the base of an upright needle. As the viscin dries, it becomes solid and cements the seed to the shoot surface. Only a small fraction of the seeds that land on susceptible hosts are transferred to the twigs; most seeds fall off the host. In the cases of *A. americanum* and *A. vaginatum*, an average of 20% of the seeds that landed on a host needle was transferred to twigs (Hawksworth 1965).

Dwarf mistletoe seeds must remain attached to the host over winter. In the spring, the seed germinates and develops a radicular apex. Once this structure comes in contact with an obstruction, such as a needle base, a holdfast is formed. The centre of the holdfast

develops a penetration wedge that forces into the host cortex (Scharpf and Parmeter 1967). The penetration structure appears to crush or push host cells out of the way as it enters the host (Hunt, Owens and Smith 1996).

Once the host periderm has been penetrated, the host branch is invaded. An incubation period of 2-5 years elapses before the first shoots appear. One to two years after shoots develop, the dwarf mistletoe infection may begin to flower. Flowering typically lasts 4-6 weeks and, in most cases, occurs during annual flowering periods. There are a few examples of tropical species of dwarf mistletoe, such as *Arceuthobium aureum* sp. *aureum*, which appear to flower continuously throughout the year. After pollination, fruits mature on the female infection the following year and the cycle is repeated (Hawksworth and Wiens 1996).

Dwarf mistletoes live on the living tissue of the host tree and can survive as long as the host branch is alive (Anonymous 1985). As the host grows, dwarf mistletoe infections on the lower branches may be shaded out, lose all their shoots and become dormant. Gill and Hawksworth (1961) have suggested that dwarf mistletoe may live indefinitely in the dormant state. These dormant dwarf mistletoe infections can be reactivated if conditions improve. Increasing light levels through selective logging, for example, may reactivate dormant dwarf mistletoe infections (van der Kamp 1986).

1.2.4 Biology of dwarf mistletoe

The dwarf mistletoe infection can be separated into two parts: the shoots and the root-like system that forms in the host tissue. The shoots are the stem-like portions of the dwarf mistletoe infection outside the host that function primarily in reproduction (Baranyay, Hawksworth, and Smith 1971). Although dwarf mistletoe shoots are capable of some photosynthesis, the parasite derives a large proportion of its photosynthate and all of its water and minerals from the host (Leonard and Hull 1965). Dwarf mistletoes are therefore considered hemiparasitic, or "capable of some autotrophic carbon fixation, but dependent on the host for an unknown quantity of reduced carbon compounds." (Miller and Tocher 1975).

The root-like portion of the dwarf mistletoe infection that forms in the host tissue has been termed the endophytic system (Thoday and Johnson 1930). Depending on the

dwarf mistletoe species, the host, and possibly the site of original infection, the endophytic system can be localized or systemic (Kuijt 1960; as cited in Hawksworth and Wiens 1996). It has also been suggested that the site of inoculation, whether in primary or secondary tissue, has a significant impact on the form of endophytic system that develops for those dwarf mistletoe species capable of systemic infection (Alosi and Calvin 1984).

In localized infections, the dwarf mistletoe endophytic system can only keep up with cambial growth and is contained within a limited section of the host branch. The endophytic system often causes swelling of the infected host branch, and the shoots remain localized near the original site of infection. In systemic infections, the endophytic system keeps pace with apical and cambial growth of the infected branch. The dwarf mistletoe endophytic system ramifies extensively through the host tissue of broom branches arising from infected bark, and swelling of the host branch is not so pronounced. The shoots of systemic infections are scattered along the host branch or concentrated at branch nodes. Few dwarf mistletoes are capable of systemic infection, and it has been argued that systemic infection is more evolutionarily advanced (Hawksworth and Wiens 1972). Most dwarf mistletoes are only able to infect secondary tissue and have limited ability to move distally along the host branch (Alosi and Calvin 1984).

The endophytic system of dwarf mistletoes differs from typical plant roots in that it lacks a root cap, pericycle, endodermis, and continuous phloem and xylem (Cohen 1954; Alosi and Calvin 1985; Calvin, Hawksworth, and Knutson 1984). Beginning with a primary haustorium at the infection site, a localized infection develops haustorial (cortical) strands, which extend longitudinally and sometimes circumferentially through the host cortex and outer host phloem (Alosi and Calvin 1984). Other structures termed "sinker" have radially oriented growth and extend from the haustorial strands into the host secondary vasculature. Although given a different name, sinkers are not considered to be distinct organs of the haustorial system; rather, the name is used in reference to radially oriented portions of the endophytic system or tissue types (Alosi and Calvin, 1984).

Sinker tissue is almost always associated with host rays (Thoday and Johnson 1930; Cohen 1954; Srivastava and Esau 1961). If young sinkers develop in a region

where ray cells do not occur, new ray cells quickly develop in relationship with the sinker cells. The resulting structure, composed of radially-orientated sinker cells surrounded by host ray cells, has been termed an "infection ray" (Srivastava and Esau 1961). The infection ray may consist of host ray parenchyma, host albuminous cells, sinker ground parenchyma, sinker xylem, and sinker sheath cells.

The structure of the systemic endophytic system differs significantly from the localized endophytic system in the primary growth region of the host. In the case of *Arceuthobium douglasii*, no sinkers or multiseriate haustorial strands develop in the primary endophytic form. The parasite cells do not specialize in this region, and only the inner cortex, phloem, and procambium are invaded. Once the host branch matures and starts to develop secondary tissue, the endophytic system of *A. douglasii* changes. The primarily uniseriate strands of the mistletoe develop into more complex, multiseriate, longitudinal strands that cease elongation and expand radially. Apical strands of the parasite grow towards the cambium, and sinker cells develop, resulting in an endophytic structure that is more similar to that of localized infections (Alosi and Calvin 1984).

Possibly as a result of continual host-parasite association, systemic infections tend to have less anomalous wood when compared to localized swellings. For example, infection of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) by *A. douglasii* did not alter the size of the growth rings of the host, or induce traumatic resin ducts, which are common in localized dwarf mistletoe swellings (Alosi and Calvin 1984). However, abnormal longitudinal shrinkage and twisting of the wood on drying may still occur as a result of increased microfibril angle in tracheids in infected wood (B. J. van der Kamp, University of British Columbia, Vancouver, BC, pers. comm.).

Due to their location within the host, sinkers are believed to be associated with parasite nutrient uptake (Alosi and Calvin 1984). The transfer of water and minerals, host-originating photosynthate, and possibly organic compounds from the host to the dwarf mistletoe occurs in the common apoplast (Alosi and Calvin 1985). Tainter (1971) suggested that conversion of carbohydrates into lipid would create a sufficient concentration gradient to maintain flow of carbohydrates from the host to the dwarf mistletoe infection. Once absorbed, these are then carried through the symplast of the parasite to the shoots.

The exact transport method of nutrients from the dwarf mistletoe endophytic system to the shoots is not understood. Depending on the parasite species, phloem and xylem are reduced or absent in the dwarf mistletoe endophytic system and shoots (Calvin, Hawksworth, and Knutson 1984; Alosi and Calvin 1984). A possible route for nutrient mobilization from the endophytic system to the shoots may involve coupling diffusion potentials to water pressure (Alosi and Calvin 1985).

1.2.5 Distribution of dwarf mistletoe

Of the 42 species of dwarf mistletoe, 34 occur in the New World. Only one species (*Arceuthobium juniper-procerae*) occurs in the southern hemisphere. In the new World, dwarf mistletoes are found from southeastern Alaska, northern Canada, and Newfoundland through most of the western United States and Mexico to Central America (Honduras), as well as in an outlying population on the island of Hispaniola. Greatest species diversity occurs in northwestern Mexico and the western United States. Of all the species in the New World, *Arceuthobium douglasii* has the widest distribution, ranging from southern British Columbia (BC), Canada, southward throughout most of the western United States to southern Durango, Mexico (Hawksworth and Wiens 1996).

Nineteen species of dwarf mistletoe occur in the United States, and 5 of these occur in Canada. These include *Arceuthobium americanum*, *A. douglasii*, *A. laricis*, *A. pusillum*, and *A. tsugense*. All but *A. pusillum* are found in BC (Hawksworth and Wiens 1996).

1.2.6 Hosts of dwarf mistletoes

The hosts of dwarf mistletoe are commonly ranked according to susceptibility to infection using the categories "principal", "secondary", "occasional", and "rare" (Hawksworth and Wiens 1972). Conifers that are not parasitized by a particular dwarf mistletoe species are categorized as "immune". Hawksworth and Wiens (1972) designed these classifications based on an infection index, which is defined by percentage classes of infected trees for each host species within 6 m of a heavily parasitized host. Principle hosts have infection levels that are greater than 90%, secondary hosts between 50-90 %,

occasional hosts between 5-50%, and rare host between 0-5%. Immune species are expected to have 0 % infection levels.

The principal hosts for dwarf mistletoes worldwide are typically restricted to a single genus or for *Pinus* to a single subgenus. Only *Arceuthobium microcarpum*, *A. laricis*, and *A. tsugense* have principle hosts belonging to different genera. These are *Picea* and *Pinus*; *Larix* and *Tsuga*; and *Abies*, *Pinus* and *Tsuga*, respectively (Hawksworth and Wiens 1996).

In the New World, the principal hosts of dwarf mistletoe include *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, and *Tsuga*. In the Old World, principle hosts are *Abies*, *Keteleeria*, *Juniperus*, *Picea*, and *Pinus*. Host specificity of dwarf mistletoe species is broad, ranging from a single primary host, such as in the case of *Arceuthobium apachecum* on southwestern white pine (*Pinus strobiformis* Engelm.), to several primary hosts. Occasionally, artificial inoculation may result in infection of tree species that are not considered to be natural hosts.

In Canada, the trees most seriously affected by dwarf mistletoe are lodgepole pine (*Pinus contorta* Dougl. ex Loud.), Jack pine (*P. banksiana* Lamb.), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), western larch (*Larix occidentalis* Nutt.), and black spruce (*Picea mariana* (Mill.) BSP); (Hawksworth and Wiens 1996). In BC, dwarf mistletoes attack hemlock on the coast, lodgepole pine in the interior and Douglas fir and larch in the southeastern interior (Anonymous 1985). In the western United States, the most seriously damaged trees are ponderosa pine (*Pinus ponderosa* P. Laws Ex C. Laws), lodgepole pine, Jeffrey pine (*P. jeffreyi* Grev. & Balf.), Douglas fir, California red fir (*Abies magnifica* Dougl. ex D. Don), white fir (*A. concolor* (Gord. & Glend.) Lindl. ex Hildebr.), western larch, and western hemlock (Hawksworth and Wiens 1996). Cedar, juniper, cypress, redwood, and giant sequoia appear to be immune to dwarf mistletoe infection in North America (Johnson and Hawksworth 1985).

1.2.7 Impact of dwarf mistletoe in forestry

Dwarf mistletoes are the single most destructive pathogen of commercially valuable coniferous timbers in several regions of Mexico, western Canada, western United States, and parts of Asia (Hawksworth and Wiens 1996). It has been estimated

that dwarf mistletoes cause economic losses amounting to 11.3 million cubic meters of wood annually in the western United States, and 3.8 million cubic meters in western Canada (Drummond 1982; Sterner and Davidson 1982; as cited in Hawksworth and Wiens 1996).

In cases of high infection, both the height and diameter growth rate of infected trees can be reduced. In one study, lodgepole pine heavily infested with dwarf mistletoe were on average 6-8 feet shorter in total height when compared to non-infected trees (Dobie and Britneff 1975). In another study, Hawksworth and Hinds (1964) observed a 5% cumulative reduction in diameter growth per decade for lodgepole pine (as cited in Hawksworth and Johnson 1989).

Although the mechanism by which dwarf mistletoe reduces tree growth is not yet fully understood, it is believed that dwarf mistletoe infections alter the host metabolic balance, making the site of infection a nutrient sink (Leonard and Hull 1965). In this way, dwarf mistletoe infections reduce the amount of host nutrients available for host growth and development, thus reducing host vigour.

Infection of a conifer by dwarf mistletoe may also reduce cone and seed production, and predispose the host to infection by other decay fungi, insects, and environmental factors (Johnson and Hawksworth 1985; Hawksworth and Johnson 1989). Under severe conditions of infection, dwarf mistletoe can kill the host. The rate at which an infected tree dies depends on its age when it was first infected and the number of infections. Older trees are slower to show measurable effects of dwarf mistletoe infection (Johnson and Hawksworth 1985).

1.2.8 Spread and intensification of dwarf mistletoe

The distribution and intensity of dwarf mistletoe infections in young, regenerating stands depends on the distribution of residual trees, the disease within the residual tree, and the distance between the residual tree and the susceptible host (Smith 1977). In some cases, removal of the original residual tree after infection has established in a regenerating stand does not change the pattern of infection, indicating that internal re-infection of the regenerating stand greatly influences the pattern of dwarf mistletoe spread (Smith 1977).

The growth rate of dwarf mistletoe is hypothesized to be faster on vigorous hosts when compared to suppressed hosts. *Arceuthobium americanum* on lodgepole pine and *A. vaginatum* f. sp. *cryptopodum* on ponderosa pine both formed new shoots nearly twice as rapidly on vigorous hosts when compared to suppressed hosts (Hawksworth 1960).

In dense stands, dwarf mistletoe infections in the middle and upper crown are most damaging. The occurrence of mistletoe in the lower crown region is of less concern because the relative spread is small and the infections may be shaded out (van der Kamp 1986). In addition, older dwarf mistletoe infections are more susceptible to attack by secondary pathogens and mechanical damage.

Simulation models of dwarf mistletoe infection and spread have been developed for lodgepole pine, ponderosa pine, and western hemlock stands (Bloomberg, Smith, and van der Wereld 1980). More recent models include the Dwarf Mistletoe Spread and Intensification model (DMSI) developed by ESSA in cooperation with the USDA Forest Service and the Dwarf Mistletoe Impact Modeling System (DMIM), which was designed to run in conjunction with the Forest Vegetation Simulator (FVS, also known as PROGNOSIS); (Hawksworth et al. 1995). These models are important for estimating the long-term impact of different control strategies and are used to determine which control strategy will work best under certain conditions.

1.3.0 Management strategies for dwarf mistletoes

Dwarf mistletoe has been associated with extensive timber loss in many parts of the world, particularly in North America. Because of this, effort has been made to develop efficient control methods for this parasite. Management of dwarf mistletoe within a stand requires detection, evaluation, prevention, and suppression of the pathogen (Johnson and Hawksworth 1985).

Because of the life cycle and small size of dwarf mistletoe, it is not always easy to detect early stages of infection in the field. This is especially true when the shoots have not yet developed. In addition, various stem deformities or accumulation of organic debris may be misidentified as infections, and high up infections may be hidden by dense foliage and difficult to detect (Shaw, Freeman, and Mathiasen 2000). In a study of the accuracy of ground-based hemlock dwarf mistletoe rating, one observer rated 98% of the

trees having infection, when in fact a crane survey estimated only about 53% of the trees having infection (Shaw, Freeman, and Mathiasen 2000). Other observers underestimated the amount of infection in the field, indicating that there is a range in the accuracy of ground-based dwarf mistletoe rating and often strong observer bias. In general, the easiest indication of dwarf mistletoe infection is the presence of shoots or basal cups. A polymerase chain reaction (PCR) technique designed to detect early dwarf mistletoe infection within the host branch has recently been developed (Marler et al. 1999) and may prove useful in detecting dwarf mistletoe infections before visible signs of infection become established.

Despite some difficulties in detecting dwarf mistletoe in the field, numerous dwarf mistletoe-rating systems have been developed. Hawksworth and Lusher (1956) produced a 6-class rating system for dwarf mistletoe infection (as cited in Hawksworth 1977). The 6-class rating system is used to quantify the degree of infection so that stand management priorities can be established, and to quantify the mistletoe-infection hazard of overstory trees or stands to understory stands. In addition, the rating system helps estimate growth loss and mortality and define which trees are suitable for seed trees (Hawksworth 1977).

The first step for determining the disease rating of an infected tree involves dividing the live crown into thirds. Each third is then rated as: 0, no mistletoe, 1, light mistletoe (less than half of the branches infected) or 2, heavy mistletoe (more than half the branches infected). As a last step, the ratings of each third are added together to give a final disease rating for the tree. Although it was observed that field personnel have a bias to either over or under estimate the true dwarf mistletoe rating, the 6-class rating system is still useful for predicting management implications and understanding the biology of dwarf mistletoe (Shaw, Freeman, and Mathiasen 2000).

Prevention is much a more effective means of control than removing dwarf mistletoes after they have infected a particular stand. With proper management of tree stands, it is possible to reduce the spread of dwarf mistletoe to nearby cut blocks. For example, designing treatment blocks that take advantage of natural or man-made barriers (i.e. rivers, roads) will reduce the spread of dwarf mistletoe from nearby infected stands (Johnson and Hawksworth 1985). It is also advisable to use clear cuts 20 acres (8 ha) or

larger in infected stands, and to make sure regenerating stands are disease free. The use of resistant species may also reduce dwarf mistletoe spread and intensification, and maintaining rapid tree growth can sometimes allow hosts to outgrow dwarf mistletoe infection (Richardson and van der Kamp 1972).

Ideal strategies for management of forest pests should be cost effective, efficacious, environmentally sound, and sustainable. Prior to logging, forest fires were the main way in which dwarf mistletoes were eradicated from diseased stands (Alexander and Hawksworth 1975). Fire sanitized dwarf mistletoe infected stands by killing the host (and the parasite) and changing forest composition (Johnson and Hawksworth 1985). Once logging became established, silvicultural control of dwarf mistletoe was most commonly used. Today, alternative methods of control for dwarf mistletoes, such as chemical, genetic, and biological control are presently being sought (Shamoun and DeWald 2002).

1.3.1 Traditional control of dwarf mistletoes

Dwarf mistletoes are ideal candidates for silvicultural management because they require a living host, have high host specificity, have a long life cycle, spread relatively slowly, and are easy to identify in the field when signs of disease are present (Johnson and Hawksworth 1985). Silvicultural control can be considered a natural means of management that uses preventative and suppressive mechanisms. Because of the relatively long life cycle of dwarf mistletoes, it can take many years and several generations of dwarf mistletoe infection before levels reach damaging proportions (Scharpf and Parameter 1967). If silvicultural control is successful and dwarf mistletoe is removed from a stand during the first rotation, then dwarf mistletoe should not be a serious problem for the subsequent rotations (Baranyay and Smith 1977). Prevention and removal of infected trees can therefore be sufficient in many cases for control. Occasionally, selective burning of infected trees can also be employed as a control strategy for dwarf mistletoe (Johnson and Hawksworth 1985).

Due to the high variability of dwarf mistletoe disease incidence, a suitable control method must be determined on a case by case situation and take into account stand composition, stand age, number of years to harvest, disease incidence and pattern and

length of time the stand has been infected (Baranyay and Smith 1977). In addition, acceptable forest practices for the region of interest must be considered.

The most effective way to control dwarf mistletoe is to remove infected trees at harvest, particularly taller infected trees that may spread infection more rapidly (Anonymous 1985). This silvicultural approach uses either clearcutting or selective removal of diseased trees to remove dwarf mistletoe from a stand.

Clearcutting is arguably one of the best ways to control dwarf mistletoe in that it often removes all susceptible trees from a stand, thereby eliminating dwarf mistletoe infection. In some instances, small advance vegetation that was not removed during clearcutting may have residual dwarf mistletoe infection and require further treatment. Larger cut block sizes in infected stands are superior to smaller cut sizes because they reduce the ratio of edge residual trees to regenerating stands (Johnson and Hawksworth 1985; Buckland and Marples 1952). In many cases, clearcutting can lead to total and long term control of dwarf mistletoe.

An alternative to clearcutting is selective removal of dwarf mistletoe infected trees, which can be integrated into the logging operations and typically involves mechanical removal or in some cases burning of the infected trees. However, there are several disadvantages to using selective removal as a means to control dwarf mistletoe. Because of the latency period in most dwarf mistletoe life cycles, detection and complete eradication of this disease using sanitation cutting and pruning is a virtual impossibility (Scharpf and Parameter 1967). In addition, intermediate cutting of dense dwarf mistletoe infected stands may reactivate dormant mistletoe infections by increasing light within the stand (Richardson and van der Kamp 1972; van der Kamp 1986). Thinning a stand may also reduce obstructions to dwarf mistletoe seed dispersal. These factors combined may temporarily increase the rate of upward advance and intensification of dwarf mistletoe within the stand (Richardson and van der Kamp 1972).

Current forest practices in BC (online at <http://www.for.gov.bc.ca/tasb/legsregs/fpc/>) make silvicultural control of dwarf mistletoe more difficult than in the past by reducing the size of clearcut blocks and increasing riparian reserves and the use of partial cutting. Research has shown that the infection level of second growth trees was proportional to the number of residual diseased trees, indicating that current forest

practices may favour the spread of dwarf mistletoe (Bloomberg and Smith 1982). Furthermore, silvicultural control of dwarf mistletoe is sometimes not possible. For example, it is not always preferable to mechanically remove dwarf mistletoe infected trees from parks, watersheds, or riparian zones (Hawksworth and Wiens 1996). Because of these difficulties, alternative methods for controlling dwarf mistletoes are presently being investigated. These include chemical, genetic, and biological control.

1.3.2 Chemical control of dwarf mistletoes

Chemical control is advantageous in that it does not require mechanical removal of the host and is less labour intensive than silvicultural control. Chemical control of dwarf mistletoes in ornamental landscapes is especially alluring because the only alternative method for control, pruning, is expensive, laborious and reduces the aesthetics of the host (Adams, Frankel, and Lichter 1993). However, there has been little success in developing a chemical herbicide for dwarf mistletoe.

Nearly 60 different chemicals were tested against dwarf mistletoe in the 1960's and 1970's, but none proved effective in killing the dwarf mistletoe infection without harming the host (Johnson, Hilderbrand, and Hawksworth 1989). In some cases, application of test herbicides damaged the host tree and had little impact on the dwarf mistletoe infection (Quick 1964). Today, many of these chemicals are considered unsafe and have been removed from the market.

Ethephon [(2-chloroethyl) phosphoric acid] is the most promising chemical herbicide for dwarf mistletoe (Hawksworth and Johnson 1989). Ethephon is registered in the US under the trade name Florel® but is not registered in Canada. Its mechanism of action involves the release of ethylene during absorption by plant tissue, which speeds up the natural ripening process and results in abscission of dwarf mistletoe shoots. Shoot abscission rates for *Arceuthobium vaginatum* treated with ethephon ranged from 73-98 % (Johnson, Hilderbrand, and Hawksworth 1989). Ethephon does not kill the dwarf mistletoe endophytic system, however, and new dwarf mistletoe shoots may develop from the dwarf mistletoe swelling. Retreatment may therefore be required.

Although ethephon does not kill the dwarf mistletoe infection, it may still be effective in reducing dwarf mistletoe in the field. Applying ethephon three to five years

after thinning a dwarf mistletoe infected stand may lessen the spread and establishment of dwarf mistletoe long enough for the stand canopy to close in, thereby shading out any remaining dwarf mistletoe infections (van der Kamp 1986).

Unfortunately, there are as of yet some unresolved problems with using ethephon in the field. Toxicity of ethephon has been observed in some host trees (Adams, Frankel, and Lichter 1993). Needle chlorosis and branch dieback occurred in black spruce and lodgepole pine after treatment with ethephon (Livingston and Brenner 1983; Frankel and Adams 1989). In addition, there is growing public resistance to the application of chemical herbicides in the field (Jobidon 1991).

1.3.3 Genetic control of dwarf mistletoes

Breeding for resistance is applicable to situations where exotic pathogens are causing disease in trees that have not naturally evolved resistance or to native pathogens that have been intensified through silviculture. In addition, breeding for resistance is appropriate for situations where a high level of natural resistance to a native pathogen has failed to develop (Roth 1974). Dwarf mistletoes apply to the last of these conditions.

There are several criteria to consider when searching for a resistant host. Smith, Wass, and Meagher (1993) suggest that both the frequency and the vigour of dwarf mistletoe infection should be considered when searching for resistant western hemlock stock. In addition, inoculation procedures testing for resistance should be consistent in the age of branch segment, branch order, and positioning of the seeds along each segment.

Because of the slow rate of spread and uneven distribution of dwarf mistletoe in the field, development of dwarf mistletoe host resistance is not as advanced as that of other hosts with air-borne, more evenly distributed pathogens such as foliage diseases and stem rusts (van der Kamp 1991). The unequal exposure to seed source and infection variables makes it difficult to detect resistant varieties in the field (Roth 1974).

As a result of the difficulties in detecting resistant hosts in the field, inoculation trials have been used to screen for host resistance to dwarf mistletoe. Besides having fewer or no dwarf mistletoe infections, resistant hosts may have slower dwarf mistletoe endophytic development or fewer dwarf mistletoe shoots (Smith 1974). For example, infection of larch and Douglas fir by *Arceuthobium tsugense* resulted in slow endophytic

development (Smith 1974). Only 1 of 38 western larches infected by *A. tsugense* produced shoots, and shoot development was slower than that observed for susceptible hosts.

Studies screening for variation in resistance against dwarf mistletoe infection have been much more rigorous between host species than within-host species (Shamoun and DeWald 2002). Research supports the existence of natural within-host species resistance to dwarf mistletoe infection. One inoculation trial found western hemlock and ponderosa pine varied in their susceptibility to *Arceuthobium tsugense* and *A. campylopodum*, respectively (Smith, Wass, and Meagher 1993). Within-host species resistance has also been observed in *Arceuthobium vaginatum* subsp. *cryptopodum* on ponderosa pine, *A. campylopodum* on ponderosa pine and Jeffery pine (*Pinus jeffreyi* Greville & Balfour), and *A. chinense* on Evelyn keteleeria (*Keteleeria evelyniana* Mast.); (Bates 1927; Roth 1953; Scharpf 1984; Tong and Ren 1980; as cited in Hawksworth and Wiens 1996).

Resistance mechanisms may occur at any stage of the dwarf mistletoe life cycle and range from a reduction in seed germination to apparent host immunity (Smith 1974). The ability of dwarf mistletoe seeds to remain attached to a host and penetrate the host surface is the first point at which some resistance may occur. Conifers with drooping or open foliage, such as western larch and pines, have been shown to have low dwarf mistletoe seed retention (Smith 1974). In addition, selection for shorter needle bases may reduce the penetration leverage of germinated dwarf mistletoe seeds and reduce the rate of successful infection (Hunt, Owens, and Smith 1996).

Host response to infection may also impart resistance. In the case of red firs, penetration by *Arceuthobium campylopodum* f. sp. *abietinum* seeds was resisted by the development of an abscission zone at the base of the needle (Scharpf and Parmeter 1967). The abscission zone was followed by the development of wound cork, which inhibited penetration by the parasite. In another study, Tainter and French (1971) observed the formation of wound periderm in response to infection of eastern larch (*Larix laricina* (DuRoi) K. Kosh.) by *Arceuthobium pusillum*.

As more research breeding and screening for genetic resistance to dwarf mistletoe infection takes place, a deeper understanding of the mechanisms involved in resistance will emerge. Recently, Nowicki, DeWald, and Moser (1999) found three neutral markers

with alleles using isozyme analysis that appear to be specifically associated with *Arceuthobium douglasii* infected Douglas fir trees. The purpose of these alleles is not fully understood, and more research into this field is required.

Resistance screening programs for Douglas fir and western hemlock are currently underway at Northern Arizona University and the Canadian Forestry Service, Pacific Forestry Centre (Cartwright et al. 2003; Shamoun and DeWald, 2002).

1.3.4 Biological control of dwarf mistletoe

Although the concept of using plant pathogens to control forest pests is not new, development of biopesticide technology is still in its infancy (Evans 1995). The biological control approach involves enhancing natural plant pathogens such that the pathogen reduces the vigour of unwanted pests. The aim of an efficient biological control strategy is not complete eradication of the pest, which may upset the ecosystem, but rather to effectively manage the pest by reducing inoculum (Shamoun 1997).

There are two types of biological control: classical control and inundative control. Classical biological control is based on the phenomenon that introduced organisms, in this case a pathogen or insect, may achieve dominance in a new environment because they will have few or no natural enemies or competitors (Evans 1995). Because of growing concern about introducing new organisms, the use of classical control is losing attractiveness. However, it is still promising as a means to control introduced organisms.

Inundative biological control, on the other hand, uses indigenous pathogens to control target forest pests. This approach involves mass production and application of inoculum on the pest such that the pathogen interferes with the pest life cycle (Evans 1995).

Only a small number of inundative biological control agents have been registered for use against forest pests. *Chondrostereum purpureum* (Pers.:Fr) is a wood-inhabiting fungus that invades the cambium and sapwood of mostly hardwood trees and shrubs. It is currently marketed as a wood decay promoter and as a biological control agent for woody weeds in Europe under the trade name BioChon™ (Ravensberg 1998; as cited in Evans, Frohlich, and Shamoun 2001). *Chondrostereum purpureum* is also registered in eastern Canada as Myco-Tech™. A collaborative research project between MycoLogic

Innovative Biologicals Inc and the Canadian Forest Service is in the process of registering a different isolate of *C. purpureum* in western Canada under the tradename Chontrol™ (S. F. Shamoun, Pacific Forestry Centre, Victoria, BC, pers. comm.).

Stumpout™ is another example of an inundative biological control agent used to control forest pests. Stumpout™ was registered in South Africa in 1997 and is currently used as a mycoherbicide to prevent re-growth of cut wattle stumps and to control introduced tree species (Morris, Wood, and Den Breeyen 1998; Lennox, Morris, and Wood 1999; as cited in Evans, Frohlich, and Shamoun 2001). It consists of the white-rot fungus *Cylindrobasidium laeve*, which is known to occur in South Africa and North America (Ginns and Lefebvre 1993; as cited in Evans, Frohlich and Shamoun 2001).

Inundative biological control can also be used to control forest pathogens. For example, the wood rotting fungus *Phlebiopsis gigantea* (Fr.) Jul. has been used to reduce the spread of the causal agent of annosus root and butt rot, *Heterobasidion annosum* (Fr.) Bref. (Rishbelth 1963). Inoculation of fresh cut pine stumps with formulated spores of *P. gigantea* reduced colonization of the stump by *H. annosum*. *Phlebiopsis gigantea* been registered in the United Kingdom as PG Suspension™ and in Finland as RotStop™ (Rishbelth 1963; Roy et al. 2003; Pratt 1999). Biological control of the root pathogen *Armillaria ostoyae* (Romagn.) Herink using *Hypholoma fasciculare* (Huds. Ex. Fr.) has also been investigated in Canada (Chapman and Xiao 2000).

Although there is potential for biological control of forest pests and pathogens, the majority of inundative biological control research has focused on weeds in agricultural systems. Because of this, more biological control agents have been registered for agricultural weeds than for forest pests and pathogens. Devine®, a formulation of *Phytophthora palmivora* (Butl.) Butl., was registered to control *Morrenia oderata* Lindl., strangler vine or milkweed vine, in citrus grooves (Ridings 1986). BioMal™ was registered in 1992 for control of round leaf mallow in Canada using *Colletotrichum gloeosporioides* Penz. Sacc. f. sp. *malvae* (Makowski and Mortensen 1992). However, due to the high cost of inoculum production, BioMal™ was never marketed (Watson et al. 2000).

Potential biological control agents for management of dwarf mistletoe must meet certain criteria. These attributes include: 1) a distribution which coincides with that of the

target pathogen, 2) ecological amplitude sufficient to assure persistence within its host range, 3) production of abundant inoculum (spores) for establishment of epiphytotics, 4) high infectivity, 5) high virulence, and 6) an efficient mode of action for curtailing development of target disease (Wicker and Shaw 1968). In addition, the ideal biological control agent should be able to cause infection without the necessity of wounding, and be easily formulated for field application and long-term storage (Shamoun 1997).

Some insects endemic to Pakistan have been identified as potential biological control agents for dwarf mistletoes (Mushtaque and Baloch 1979). Mushtaque and Baloch (1979) observed 27 insects and mites known to damage mistletoes. Six of these occurred in reasonable numbers, appeared to be restricted eaters, and caused appreciable damage to dwarf mistletoe. Although tests on the biology and host specificity of the 6 insects showed at least 3 of them are promising potential biological control agents for dwarf mistletoe, further research into their use in North America has not been done.

Numerous fungi have been isolated from dwarf mistletoe infections (Baranyay 1966; Hawksworth, Wicker, and Scharpf 1977; Kuijt 1963). Some of these fungi appear to parasitize the dwarf mistletoe shoots, while others have been isolated from diseased dwarf mistletoe swellings.

1.3.4.1 Hyperparasites of dwarf mistletoe shoots

Pathogens that attack dwarf mistletoe shoots are advantageous in their rapid disease development and direct impact on flower and berry development. These pathogens are useful in that they can reduce the ability of dwarf mistletoe to spread and intensify in the field. However, shoot pathogens may not enter the dwarf mistletoe swelling, and therefore do not eradicate the dwarf mistletoe infection (Wicker and Shaw 1968).

At least six hyperparasites are known to attack the shoots of dwarf mistletoes in North America. The most common fungi with the widest dwarf mistletoe host range are *Caliciopsis arceuthobii* (Peck.) Barr., *Cylindrocarpon gillii* (Ellis) J. A. Muir, and *Colletotrichum gloeosporioides* (Pen.z) Penz. & Sacc.. The other fungi (*Pestalotia heterocornis* Guba, *Cylindrocarpon* sp., *Pestalotia maculiformans* Guba & Zeller,

Metaspheria wheeleri Lider, and *Alternaria alternata* (Fr.) Keissler) are rare or only occur locally and will not be discussed (Hawksworth, Wicker, and Scharpf 1977).

Caliciopsis arceuthobii (formerly *Wallrothiella arceuthobii* (Pk.) Sacc.) parasitizes the pistillate flowers of *Arceuthobium pusillum* and *A. douglasii* in the United States and Canada (Peck, 1875; as cited in Wicker and Shaw 1968) as well as *A. americanum* and, rarely, *A. vaginatum* subsp. *cryptopodum* (Hawksworth, Wicker, and Scharpf 1977). The fungus is restricted to the floral organs of the pistillate shoots of dwarf mistletoes and prevents normal seed development. *Caliciopsis arceuthobii* occurs in two geographical locations: the northern location (BC, Oregon, Washington, Idaho, Montana) and the southern location (Colorado, Arizona, New Mexico, and Mexico); (Hawksworth, Wicker, and Scharpf 1977; Ramsfield 2002). The fungus has not been found in the region between these two locations. It has been suggested that *C. arceuthobii* may only be able to infect spring flowering dwarf mistletoe species (Kuijt 1963), but this remains speculation. The extent of damage caused by *C. arceuthobii* is variable, more than 90% of one year's crop of berries may be destroyed one year, and yet the next year the fungus may be absent from the same location (Hawksworth Wicker, and Scharpf 1977). A study conducted by Wicker and Shaw (1968) suggests that the amount of disease within a stand may vary from high to absent within as little as 2 years. In addition, although *C. arceuthobii* has been grown in culture, spores have not been produced *in vitro* (Knutson and Hutchins 1979; Parker 1970). These combined limit the use of this fungus as a biological control agent for dwarf mistletoe.

Cylindrocarpon gillii (formerly *Septogloeum gillii* Ellis) attacks all parts of the dwarf mistletoe shoots (Hawksworth Wicker, and Scharpf 1977; Muir 1973). Known hosts of *C. gillii* are *Arceuthobium abietinum* f. sp. *concoloris*, *A. abietinum* f. sp. *magnificae*, *A. americanum*, *A. apachecum*, *A. blumeri*, *A. californicum*, *A. campylopodum*, *A. cyanocarpum*, *A. divaricatum*, *A. douglasii*, *A. laricis*, *A. microcarpum*, and *A. tsugense* (Hawksworth Wicker, and Scharpf 1977). This fungus occurs more prevalently on the east slopes of the Cascade Mountains rather than in eastern Washington. It is also widely distributed in western parts of Canada, including BC and Alberta (Kuijt 1963). Early symptoms of infection are small, yellowish-white lesions on the shoots, which gradually enlarge, coalesce, and erupt through the epidermis,

releasing conspicuous masses of white spores (Ellis 1946). The hyphae of the fungus invade all the tissues of the shoot except for the vascular elements (Ellis 1939). The shoot is often girdled, killing the distal portion of the shoot. *Cylindrocarpon gillii* is similar to *Caliciopsis arceuthobii* in its ecological amplitude, and can vary in the amount of disease from high to absent within a couple of years (Wicker and Shaw 1968). A field trial conducted by Mielke (1959) that involved inoculating *A. americanum* with *C. gillii* found the parasite died out on four experimental plots only 3 years after inoculation. The lack of rain, which is required for spore dissemination and infection, was used to explain the reduction of *C. gillii* observed. Previous studies by Ellis (1946) showed that *C. gillii* is not a wound parasite and is able to penetrate unwounded host tissue. Abundant inoculum production in culture is also possible with this fungus.

Colletotrichum gloeosporioides parasitizes the shoots of many dwarf mistletoe species, including *Arceuthobium abietinum* f. sp. *concoloris*, *A. abietinum* f. sp. *magnificae*, *A. americanum*, *A. apachecum*, *A. californicum*, *A. campylopodum*, *A. divaricatum*, *A. douglasii*, *A. laricis*, *A. microcarpum*, *A. occidentale*, and *A. tsugense* (Hawksworth Wicker, and Scharpf 1977). The first symptoms of infection by *C. gloeosporioides* involve the development of small, necrotic lesions on the shoots. As the disease progresses, infection causes shoot dieback or blight, often resulting in girdling of the shoots. Signs of disease can be seen on the shoots, where acervuli and setae may occur. The mycelium of *C. gloeosporioides* is entirely intercellular and occurs primarily in the exterior portion of the shoot cortex. *Colletotrichum gloeosporioides* is more persistent than *Caliciopsis arceuthobii* and *Cylindrocarpon gillii* in terms of disease abundance over time. Abundant inoculum production in culture is also possible with this fungus (Wicker and Shaw 1968).

Recently, a study assessing the use of *C. gloeosporioides* as a biological control agent for lodgepole pine dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.) was conducted in the BC interior (Ramsfield 2002). Results from this trial were not able to show a significant reduction in mistletoe seed production as a result of inoculation with *C. gloeosporioides*. However, the fungus was not dismissed as a potential biological control agent for *A. americanum* and merits further investigation

Other, more location and species-specific fungi that parasitize dwarf mistletoe shoots have also been observed. For example, the brown felt fungus *Herpotrichia juniperi* (Duby) Petr. is known to colonize and kill shoots of *Arceuthobium abietinum* f. sp. *magnificae* growing on California red fir in the higher elevations of the southern cascades in California (Scharpf 1986). In this case, *H. juniperi* is not a feasible biological control agent because of its limited host range and its requirement of persistent snow pack to develop.

1.3.4.2 Hyperparasites of dwarf mistletoe swellings

Fungi that attack dwarf mistletoe swellings are advantageous in that disease symptoms are pronounced and the pathogen may possibly kill the dwarf mistletoe infection. However, as the host tree may be damaged as well, hyperparasites of dwarf mistletoe swellings must undergo additional laboratory study (Shamoun and DeWald 2002).

Cytospora abietis Sacc., is associated with dwarf mistletoe swellings and is common on California red fir and white fir parasitized by *Arceuthobium abietinum* (Scharpf 1969; Scharpf and Bynum 1975). This canker-causing fungus can invade the dwarf mistletoe swelling and kill the host branch. On occasion, *C. abietis* parasitizes non-mistletoe infected branches. Further research into the interactions between *C. abietis*, *A. abietinum*, and its host is required before evaluation of this fungus as a potential biological control agent for dwarf mistletoe can begin (Shamoun and DeWald 2002).

Resin disease has been observed on *Arceuthobium americanum* swellings on lodgepole pine. The symptoms of resin disease include excessive resin exudation from the dwarf mistletoe swelling, necrotic and discoloured bark tissue, repeated formation of necrophylactic periderms, retention of dead pine needles, and dead, resin infiltrated dwarf mistletoe shoots (Mark, Hawksworth, and Oshima 1976). Several weakly pathogenic fungi have been shown to cause resin-disease symptoms; with *Alternaria alternata* (Fries : Fries) von Keissler and *Aureobasidium pullulans* (de Bary) Arnaud most commonly isolated from diseased *A. americanum* swellings (Mark, Hawksworth, and Oshima 1976).

Neonectria neomacrospora (Booth & Samuels) Mantiri & Samuels (formerly *Nectria neomacrospora* Booth & Samuels) is also known to parasitize dwarf mistletoe swellings. This fungus has been observed as a virulent pathogen of *A. occidentale* swellings on pine, *A. abietinum* on white fir, and *A. tsugense* swellings on hemlock (Byler and Cobb 1972a; Funk and Baranyay 1973; Funk, Smith, and Baranyay 1973). *Neonectria neomacrospora* is discussed in greater detail in section 1.5.

1.4.0 Introduction to *Arceuthobium tsugense*

1.4.1 Symptoms and signs of *A. tsugense*

Hemlock dwarf mistletoe (*Arceuthobium tsugense* (Rosendahl) G. N. Jones) causes non-systemic infection of the host branch, often resulting in swelling formation. Shoots range in colour from greenish to reddish and the average shoot is 5-7 cm tall. Mature fruit occur on some female infections in late summer and early fall. The occurrence of basal cups on the dwarf mistletoe swelling is also a good sign of disease (Hawksworth and Wiens 1996).

The formation of witches' brooms is fairly common for *A. tsugense* infections on hemlock and can develop as soon as 14 years after exposure to inoculum (Smith 1977). Because of the erratic growth pattern of hemlock, it is sometimes difficult to detect low levels of *A. tsugense* infection based on symptoms alone. Although the PCR technique described by Marler et al. (1999) used primers that were designed to detect *Arceuthobium douglasii* and *A. laricis* in host tissue, it is expected that these same primers will work for *A. tsugense* and other dwarf mistletoe species.

1.4.2 Biology of *A. tsugense*

Studies monitoring *A. tsugense* seed dispersal and natural development of *A. tsugense* infection in the field have been done in Alaska, BC, and Oregon (Shaw and Loopstra 1991; Carpenter, Nelson, and Stewart 1979; Smith 1971, respectively). Field observations show that about 50% of seeds that land on foliage moved to twigs; germination on the host needles was rare (Carpenter, Nelson, and Stewart 1979). Of the *A. tsugense* seeds that landed on a susceptible host and remained attached overwinter, approximately 60-65% germinated and 6-33% established infection. Although the highest

percentage of successful infection was observed for the trial in Alaska, *A. tsugense* shoots were often broken and damaged and no seeds were produced over the 11-year trial period (Shaw and Loopstra 1991). Differences in seed germination and infection were attributed to pathogen attack, environmental conditions and self-pruning of the host branch.

Based on close observations of the wedging of the holdfast in the acute angle of the needle base, the levering off of the needle, and the crushing of host cells, Hunt, Owens, and Smith (1996) support the importance of mechanical force for germinated *A. tsugense* seeds to penetrate the host branch. No study of enzymatic activity has been performed; it is still not clear if enzymatic activity works in combination with mechanical forces.

Arceuthobium tsugense develops a localized infection on susceptible hosts. Swellings can develop as early as the first year following *A. tsugense* infection, but in most cases do not develop until the second year or later. The endophytic structure of *A. tsugense* consists of the haustorial (cortical) strands and sinkers similar to those observed in other dwarf mistletoe species. However, *A. tsugense* differs from some other species in that both the inner and outer haustorial strands bear sinkers, and the outer haustorial strands bear two distinct sheaths (Hunt, Owens, and Smith 1996). Cohen (1954) did not observe sinker cells on the outer haustorial strands of *A. campylopodum*. Tainter (1971) described only one sheath layer in *A. pusillum* Peck.. In addition, the *A. tsugense* endophytic system lacks sieve elements (Calvin, Hawksworth, and Knutson 1984). The maximal longitudinal growth rate of the endophytic system of *A. tsugense* was approximately 1.5cm/year on hemlock and 6.6 cm/year on shore pine (Smith, 1971).

Initial *A. tsugense* shoots develop in the second and third years after infection. The life span of *A. tsugense* shoots varies from less than 1 year up to 7 years, averaging 2-3 years. The average number of shoots per infection for 3-7 years after infection was approximately 10 shoots (Smith 1971).

The growth rate of healthy, undisturbed male and female shoots is the same for the first two years following emergence of the shoots (approximately 20 mm by year 1 and 45-55 mm by year 2); (Smith 1971). The growth rate decreases for both male and female shoots after 2 years, and by the third year the female shoots are typically taller than the male shoots.

Flowering typically occurs 3-4 years after infection between July-August, and seeds are dispersed in late September and early October the following year. Both male and female shoots can produce at least 3 crops of flowers, although the average is much less as many shoots die before flowering or after producing only one crop of flowers (Smith 1977). Once the seeds are discharged, the life cycle is repeated. Dwarf mistletoes are perennial and may produce more than one crop of flowers and, if female, berries.

The location of the *A. tsugense* infection within the host may influence *A. tsugense* shoot production. For light, moderate, and severe host infections, the number of shoots observed in the top portion of the crown was equal to or greater than the middle and lower portions of the crown (Smith 1969). For all classes, the proportion of infections with shoots or fresh cups was greatest for the top portion (96%) when compared to the middle portion (84%) and the lower portion (50%).

Recent work has developed a means to germinate *A. tsugense* seeds *in vitro* using Harvey's medium with varying concentrations of auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (6-benzylaminopurine); (Deeks, Shamoun, and Punja 1998; Deeks Shamoun and Punja 2001). Development of a technique to grow dwarf mistletoe *in vitro* is important because it could provide detailed information on dwarf mistletoe physiology and aid in developing chemical or biological control (Gill and Hawksworth 1961).

1.4.3 Distribution of *A. tsugense*

Arceuthobium tsugense is one of four dwarf mistletoes found in BC. The species occurs along the Pacific coast from near Haines, Alaska to central California, and is particularly prominent on the Queen Charlotte Islands, northern Vancouver Island, Texada Island, and on the mainland near Prince Rupert and Vancouver (Baranyay and Smith 1977). In regions where *A. tsugense* occurs, the parasite can often reach high levels of infection. A detailed survey of southeastern Vancouver Island found an average of 85% of pine trees over 1.4 m tall were infected with *A. tsugense* (Wass 1976). For reasons unknown, *A. tsugense* is not found in the hemlock stands in BC's Interior Wet Belt. Inoculation trials of western hemlock in the Interior Western Hemlock Zone using *A. tsugense* inoculum from the coast found that *A. tsugense* was able to establish and develop normally in the Interior (Smith and Wass 1979). The interior population of

hemlock in BC and the United States is geographically separated from the coastal population.

1.4.4 Taxonomy and hosts of *A. tsugense*

Because of its unusually broad host range, it has been recommended that *A. tsugense* be separated into three subspecies (*mertensianae*, *tsugense* and *contortae*); (Smith 1974; Wass and Mathiasen 2003). Two of the subspecies differ somewhat morphologically. The shoots of *A. tsugense* subsp. *tsugense* are 30% taller than *A. tsugense* subsp. *mertensianae*. In addition, subsp. *tsugense* flowers 1-2 weeks prior to subsp. *mertensianae* (Hawksworth and Wiens 1996). It is unclear if morphological differences are due to genetic or environmental factors.

The primary host for *A. tsugense* subsp. *mertensianae* is mountain hemlock (*Tsuga mertensiana* (Bong.) Carr.). Pacific silver fir (*Abies amabilis* Dougl. Forbes), subalpine fir (*A. lasiocarpa* (Hook.) Nutt. var. *lasiocarpa*), and noble fir (*A. procera* Rehder) have also been reported as principal hosts for *A. tsugense* subsp. *mertensianae* (Hawksworth and Wiens 1996). Whitebark pine (*Pinus albicaulis* Engelm.) is a secondary host, and western white pine (*P. monticola* Dougl.) is an occasional host (Hawksworth and Wiens 1996).

Arceuthobium tsugense subsp. *tsugense* has western hemlock as a primary host, and shore pine (*Pinus contorta* Dougl. ex Loud var. *contorta*) and Rocky Mountain lodgepole pine (*Pinus contorta* Dougl. ex Loud var. *latifolia* Engelm.) as occasional hosts. In addition, Pacific silver fir, subalpine fir, and noble fir have also been tentatively reported as principal hosts for *A. tsugense* subsp. *tsugense* (Mathiasen 1994).

Further division of *A. tsugense* subsp. *tsugense* into two races (shore pine and western hemlock) was under debate for many years. Reports did not agree on the significance of differences in morphology and geographical distribution between the shore pine and western hemlock races (Wass 1976; Wass and Mathiasen 2003). However, it was apparent that the two races differed in host affinities (Smith, 1974; Smith and Wass 1976, 1979; Wass 1976; Wass and Mathiasen 2003). Field observations by Smith and Wass (1976) found that western hemlock appeared more susceptible to infection originating from *A. tsugense* parasitizing western hemlock than to *A. tsugense*

parasitizing shore pine. Shore pine also appeared more susceptible to infection originating from *A. tsugense* parasitizing shore pine than from *A. tsugense* parasitizing western hemlock. Inoculation trials concurred with these observations (Smith and Wass 1979). Low levels of infection occurred when shore pine was inoculated with seeds from the western hemlock race. In contrast, inoculation of western hemlock with seeds from the shore pine race resulted in moderate levels of infection, but only a sub-portion of successful infections produced shoots. Based on these observations, Wass and Mathiasen (2003) elevated the shore pine race to the species level as *Arceuthobium tsugense* subsp. *contortae*. The hosts for *A. tsugense* subsp. *contortae* are shore pine, occasionally hemlock, and rarely western white pine.

1.4.5 Spread and intensification of *A. tsugense*

Arceuthobium tsugense is arguably one of the fastest spreading dwarf mistletoes in BC. The life cycle of *A. tsugense* may be completed in as little as 4 years; other dwarf mistletoe species typically take 5-6 years to complete their life cycle (Baranyay and Smith 1977). Infection rates of *A. tsugense* are highly variable and depend on environmental conditions and site productivity (van der Kamp 1986). Richardson and van der Kamp (1972) found the number of new *A. tsugense* infections per host tree can double every four years. In another study, the number of infected hemlock surrounding infected residuals increased logarithmically from 5-30 years after logging (Stewart 1976). Knowledge of the relatively short life cycle and rate of spread of *A. tsugense* is important in planning silvicultural and other control methods (Smith 1971).

The productivity of a site may influence the impact of *A. tsugense* infection on hemlock height growth. That is, there may be a critical rate of hemlock height growth above which *A. tsugense* infection no longer causes incremental loss (Richardson and van der Kamp 1972; van der Kamp 1986). In a good site, trees are expected to have a fast growth rate, and because of this may be able to outgrow *A. tsugense* infection and spread. The critical rate of height growth is expected to vary accordingly with site productivity and tree age (Richardson and van der Kamp 1972).

Stand density may influence *A. tsugense* spread and intensification. In one estimate, the vertical spread of *A. tsugense* in coastal western hemlock in southern BC

was approximated to be 30 cm/year in a dense stand and 60 cm/year in an open stand (Richardson and van der Kamp 1972). Over a 5 year period, an average of 26 new infections per tree and a vertical rate of spread of 0.58 m/year was observed for the open stand, and 43 new infections per tree and a vertical rate of spread of 0.27 m/year was observed for the dense stand. These data suggest that the rate of vertical spread and the rate of intensification may act independently of one another.

For *A. tsugense* infection of hemlock, Smith (1969) modified the 6-class rating system so that only the middle third of the hemlock live crown is classified (Hawksworth 1977). This approach was taken because the bottom portion of hemlock branches are often dead, broken, or missing, and the top portion of the tree is often partially hidden. In addition to containing the greatest number of living infections, the middle-third assessment was more effective and easier to use than the 6-class rating system for the whole-tree.

A simulation model of *A. tsugense* infection on hemlock has also been established (Bloomberg, Smith and van der Wereld 1980). The computer model DWARF was created to predict spread and intensification of *A. tsugense* under variable conditions. DWARF takes into consideration stocking, size, and spatial distribution of residual source trees and of regeneration; severity of infection of residual source trees; topographic slope; tree growth rate and environmental effects (e.g. wind or early frost) on mistletoe seed production, dispersal, and infection. When comparing predicted model data to actual data, the predicted average number of infections per tree was generally slightly lower than recorded number, but was not significantly different (Bloomberg, Smith and van der Wereld 1980). Applying the model to a broader range of conditions found the number of predicted infections averaged 107% of the number of recorded infections in residual trees and 128% of the infections in second-growth trees (Bloomberg and Smith 1982). Bloomberg and Smith (1982) concluded that the model predictions were generally compatible with recorded observations and provided an estimate of the effects of factors and their interactions on dwarf mistletoe incidence.

1.4.6 Impact of *A. tsugense* in forestry

In BC, the annual volume loss caused by dwarf mistletoe is about 4.2 million m³ of western hemlock and lodgepole pine (Baranyay and Smith 1977). Only decay fungi surpass this huge loss.

Infection of hemlock by *A. tsugense* reduces the rate of host growth and wood density (Wellwood 1956). A reduction in lumber yield is expected to occur as a result of swellings, which produce abnormal tissue, develop large knots in the wood, and introduce secondary pathogens and decay (Wellwood 1956).

Lightly *A. tsugense* infected hemlock trees may escape significant growth impact (Smith 1977). However, heavily infected *A. tsugense* hemlock trees can have significantly reduced host vigour. The growth of lightly infected trees was 84% and 41% greater in height and volume, respectively, when compared to severely infected trees over a 7-year period (Smith 1969). At 15 inches diameter, the average lightly infected tree was 14 feet taller and 14 cubic feet greater in volume when compared to severely infected trees. These observations apply to a mature hemlock stand with individuals averaging 110 years old.

One factor that contributes to reduced host vigour is the redirection and removal of nutrients and carbon compounds from the host to the *A. tsugense* infection. The ratio of CO₂ fixed by photosynthesis to CO₂ produced from respiration varied from 25-30% for *A. tsugense* shoots (Miller and Tocher 1975). The remaining carbon requirements are most likely derived from the host. Seasonal variation was also observed during the fall, winter and spring conditions, with *A. tsugense* having the highest rate of photosynthesis in the spring.

Another factor that may influence host vigour is the introduction of secondary fungi via the dwarf mistletoe infection. The dead bark and cankered regions often associated with the *A. tsugense* swelling provide an ideal environment for invading fungi, as evidenced by the amount of stain and decay at or near most cankered areas (Baranyay 1966). Studies have shown that infection of hemlock by *A. tsugense* can alter the bark moisture of invaded regions (Baranyay 1964). The extent and direction of impact on bark moisture varied depending on the age of the dwarf mistletoe infection, the time of year, and the growing site.

In one study, Bier (1959) noted that the relative turgidity of 5-10 year old bark of repressed hemlock trees was less (62-85%) than that of open grown trees of the same age (82-93%). In addition, lower branches with thinner foliage were found to have lower relative turgidity when compared to more vigorous, higher up branches. Bier (1959) observed that the canker disease caused by *Cephalosporium* spp. only developed when the relative turgidity of the bark was below the threshold value of 80%, and suggested that measurements of the relative turgidity of the bark may provide a useful index of tree susceptibility to disease.

1.4.7 Control of *A. tsugense*

Although silvicultural management is an effective means to control *A. tsugense*, current forest practices in BC are making this form of control difficult. Studies of host resistance to *A. tsugense* infection are underway, but as of yet no conclusive resistance mechanism has been found (Smith, Wass, and Meagher 1993, Cartwright et al. 2003). Chemical control of *A. tsugense* in BC is not feasible at present, as Ethephon is not registered in Canada and the use of chemical herbicides is losing public favour. While developing a biological control strategy for *A. tsugense* is still in its early stages, some promising potential biological control agents have been observed.

Fungal pathogens of *A. tsugense* have been collected from numerous locations throughout BC (Funk and Baranyay 1973; Shamoun 1997; Kope and Shamoun 2000). *Cylindrocarpon gillii* and *Colletotrichum gloeosporioides* have been isolated from diseased *A. tsugense* shoots (Kuijt 1963; Kope, Shamoun and Oleskevich 1997; Kope and Shamoun 2000). In addition, *Neonectria neomacrospora* has been isolated from diseased *A. tsugense* swellings (Funk, Smith and Baranyay 1973; Kope and Shamoun 2000).

1.5.0 Introduction to *Neonectria neomacrospora*

Neonectria neomacrospora (Booth & Samuels) Mantiri & Samuels (anamorph *Cylindrocarpon cylindroides* Wollenw.) has been found in Canada (Quebec and BC), parts of the United States (Oregon), and Europe (Norway); (Funk, Smith and Baranyay 1973; Byler, Cobb and Parameter 1972a, 1972b). Several sources have suggested the use of *N. neomacrospora* as a biological control agent for dwarf mistletoe and western gall

rust (Funk, Smith and Baranyay 1973; Byler and Cobb 1972; Byler, Cobb and Parameter 1972a).

1.5.1 Taxonomy and hosts of *N. neomacrospora*

This ascomycete fungus belongs to the class Pyrenomycetes, the order Hypocreales, and the family Nectricaceae. The teleomorph stage of *N. neomacrospora* has at least 5 synonyms. Originally named *Nectria cucurbitula* Tode ex Fr. var *macrospora* Wollenw, this species was renamed *Nectria fuckeliana* Booth after realization that the fungus had been misidentified. Later, Booth (1966) divided *Nectria fuckeliana* into two varieties, which were primarily distinguished on the basis of the size of their microconidia (Byler, Cobb and Parameter 1972b). These were *Nectria fuckeliana* Booth var *fuckeliana* (anamorph *Cylindrocarpon cylindroides* var *tenue*), and *Nectria fuckeliana* Booth var *macrospora* (Wr.) Booth (anamorph *Cylindrocarpon cylindroides* Wollenw). Ouellette (1972) raised *Nectria fuckeliana* var *macrospora* to the species level and renamed it *Nectria macrospora* (Wr.) Ouellette. This name, however, had already been given to previous fungal species, and it was changed to *Nectria neomacrospora* C. Booth & Samuels (Booth 1981).

Nectria neomacrospora was considered a part of the genus *Nectria* s. lat. until recently, when the group was broken down into several genera. Based on its *Cylindrocarpon* anamorph stage and the morphology of its perithecia, *N. neomacrospora* was moved into the new genus *Neonectria* (Rossman et al. 1999). Molecular work using mitochondrial ribosomal DNA sequencing supported the *Neonectria* genus as monophyletic (Mantiri et al. 2001). The name of this fungus was changed accordingly to *Neonectria neomacrospora* (Booth & Samuels) Mantiri & Samuels (Mantiri et al. 2001).

Nectria fuckeliana var *fuckeliana* is a wound parasite of Pinaceae, including *Abies*, *Larix*, and *Picea* and should not be confused with *Neonectria neomacrospora* (Booth 1979a). Some symptoms of infection by *N. fuckeliana* var *fuckeliana* are similar to those observed for *N. neomacrospora* and include die back from the tip of infected branches, production of resinous swellings, cankering of the host bark, and in some cases death of the host tree (Lang 1981).

In addition, the size of perithecia and ascospores of *N. fuckeliana* and *N. neomacrospora* overlap (Table 1.1). *Nectria fuckeliana* also occurs in the same geographical regions as *Neonectria neomacrospora* (Europe and North America).

Cultures of *Neonectria neomacrospora* may be separated from *Nectria fuckeliana* based on the size of microconidia (Table 1.1); (Booth 1979b). Hosts also differ somewhat between the two species. Although both varieties parasitize *Abies*, only *N. neomacrospora* parasitizes *Tsuga* and *Pinus* (Funk 1981).

Table 1.1 Morphology and host ranges of *Nectria fuckeliana* var *fuckeliana* and *Neonectria neomacrospora* (modified from Funk 1981; Booth 1979a, 1979b).

Species	Anamorph	Perithecia (approx.)	Ascospores	Microconidia	Hosts
<i>Nectria fuckeliana</i> var <i>fuckeliana</i>	<i>Cylindrocarpon cylindroides</i> var <i>tenue</i>	300-400 μm	13-16 X 5-6 μm	4-7 X 2.5-3 μm	<i>Abies</i> , <i>Larix</i> , <i>Picea</i> , other
<i>Neonectria neomacrospora</i>	<i>Cylindrocarpon cylindroides</i>	400-600 μm	16-22 X 5-7 μm	8-12 X 4-6 μm	<i>Tsuga</i> , <i>Pinus</i> , <i>Abies</i>

Neonectria neomacrospora has been reported as a canker pathogen on Pinaceae and balsam fir (*Abies balsamea* L. Mill.); (Booth 1979b). In other studies, *N. neomacrospora* has been isolated from swellings caused by several species of dwarf mistletoe and rust galls. In California and Washington, *N. neomacrospora* has been observed on *Arceuthobium occidentale* swellings on Bishop pine (*Pinus muricata* Dougl. ex D. Don) and *A. abietinum* swellings on white fir (Byler and Cobb 1972). In BC, *N. neomacrospora* has been observed on *A. tsugense* swellings on western hemlock (Funk, Smith, and Baranyay 1973). *Neonectria neomacrospora* is also known to infect and kill galls caused by *Peridermium harknessii* in the western United States (Byler and Cobb 1972). In the cases of dwarf mistletoe and rust gall infections, *N. neomacrospora* appeared to be specific to the diseased swelling region (Funk and Baranyay 1973; Byler, Cobb and Parameter 1972a, 1972b).

Although some studies have categorized *N. neomacrospora* as a conifer pathogen (Ouellette and Bard 1966; Ouellette 1972; Booth 1979b), others have shown *N.*

neomacrospora to be specific to diseased conifer swellings and only mildly pathogenic to healthy conifer host tissue (Byler and Cobb, 1972, Funk, Smith, and Baranyay 1973). One possible explanation for the differences in pathogenicity observed for *N. neomacrospora* is the occurrence of different races (Ouellette 1972). Ouellette (1972) suggested that *N. neomacrospora* strains from Quebec were much more virulent on *Abies* than other isolates from BC and Europe. Alternatively, due to the complex taxonomy of this species, misidentification of the fungus may have occurred.

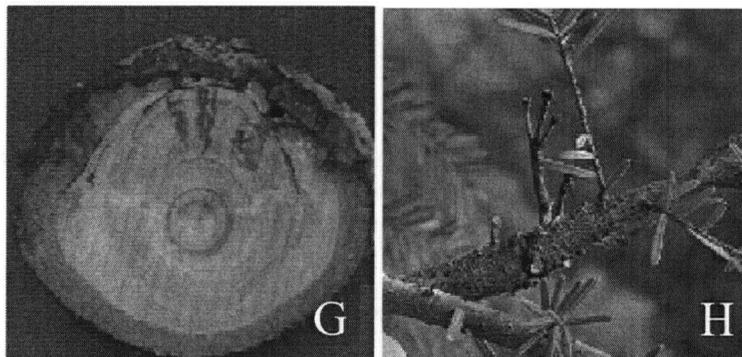
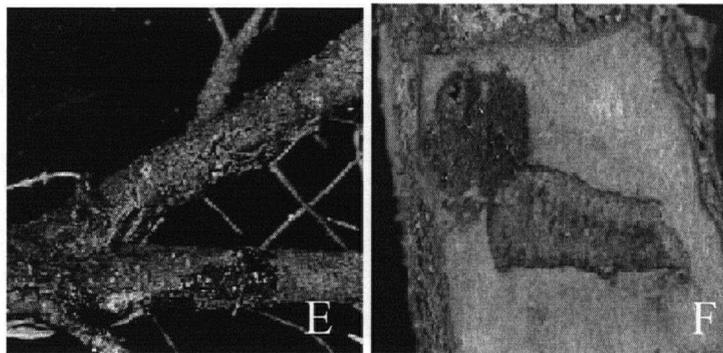
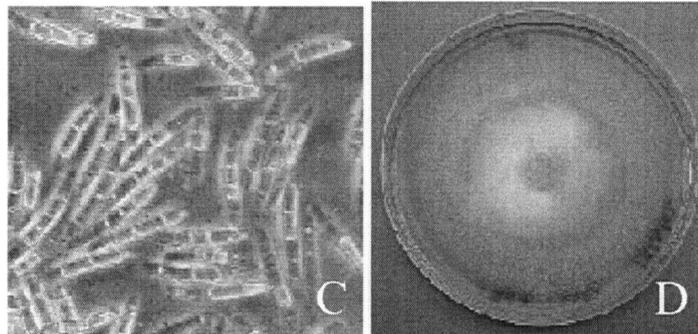
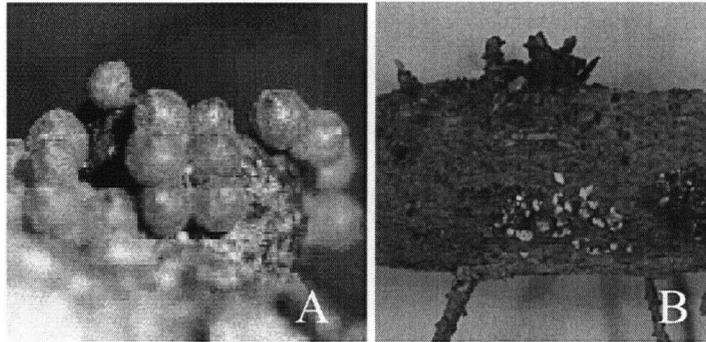
The *N. neomacrospora* isolates used in the phylogenetic study conducted by Mantiri et al. (2001) were isolated from Douglas fir rather than dwarf mistletoe swellings. However, the co-author Dr. Gary Samuels later confirmed the identity of several fungal isolates taken from *A. tsugense* swellings on western hemlock to be *Neonectria neomacrospora* (USDA-ARS Systemic Botany and Mycology Lab, Beltsville, USA; pers. comm.).

1.5.2 Symptoms and signs of *N. neomacrospora*

Typical symptoms and signs associated with *N. neomacrospora* are shown in Figure 1.1. Infection of dwarf mistletoe by *N. neomacrospora* is often associated with resinosis, sunken cankers, and, in some cases, girdling and death of the infected branch (Funk, Smith and Baranyay 1973; Smith and Funk 1980). Similar symptoms were observed for *N. neomacrospora* infected rust galls caused by *Peridermium harknessii* (Byler, Cobb, and Parameter 1972a, 1972b). Other symptoms of this fungus include dead or significantly fewer and smaller dwarf mistletoe shoots, shorter maximal distance between dwarf mistletoe shoots, and a slower rate of swelling and elongation of the dwarf mistletoe endophytic system (Smith and Funk 1980).

Figure 1.1. Typical signs and symptoms of *Neonectria neomacrospora*. A. Perithecia from the sexual stage of *N. neomacrospora*, B. Sporodochia from the asexual stage of *N. neomacrospora*, C. Macroconidia from the asexual stage of *N. neomacrospora*, D. Mycelium of the asexual stage of *N. neomacrospora* grown on potato dextrose agar for 21 days, E. Resinosis and cankering of the *A. tsugense* swelling, F. Destructively sampled *A. tsugense* swelling with top layer of bark removed to show bark necrosis, G. Cross

section of an *A. tsugense* swelling showing bark necrosis, H. Diseased and dead *A. tsugense* shoots. Pictures A and E courtesy Dr. Simon Shamoun, Pacific Forestry Centre, Victoria, BC (2003).



In one study conducted by Funk, Smith and Baranyay (1973), infection of *A. tsugense* swellings by *N. neomacrospora* resulted in an average reduction of 30 % in the number of *A. tsugense* shoots when compared to the control. Forty-four percent of the *A. tsugense* swellings colonized by *N. neomacrospora* were void of shoots (compared to 16% for the uncolonized *A. tsugense* swellings). The growth rate of *N. neomacrospora* along the *A. tsugense* swelling on potted hemlock was 39 mm/year, approximately the same rate of growth as the *A. tsugense* swelling itself (Funk, Smith and Baranyay 1973).

Signs associated with infection of *A. tsugense* swellings by *N. neomacrospora* include perithecia (perfect stage) and sporodochia (imperfect stage). Perithecia are small, reddish-brown, oval to conical ascospore producing structures that develop on the surface of dwarf mistletoe swellings. These structures measure 400-600 μ m and can be found throughout the year in the field. The ascospores are 1-septate and measure 16-22 X 5-7 μ m. Perithecia of *N. neomacrospora* can be produced in the laboratory from single spore crosses (Byler, 1970).

Sporodochia are conidia-producing structures that resemble a small mass of mycelium on the dwarf mistletoe swellings. These white, irregular structures are most commonly found in the field on *N. neomacrospora* infected *A. tsugense* swellings in the fall (Funk, Smith and Baranyay 1973). The imperfect stage of *N. neomacrospora* has characteristic macro- and microconidia that can be cultured *in vitro*. Macroconidia are predominately straight or slightly curved, 3-7 septate spore structures and measure 33-85 X 4-7 μ m. Microconidia are aseptate, oval to cylindrical spores formed from loose-branched conidiophores and measure from 8-12 X 4-6 μ m (Booth 1979b).

Both life stages of *N. neomacrospora* have been observed in the field. A study conducted by Byler, Cobb and Parameter (1972b) that made crosses of single spore isolates of *N. neomacrospora* on PDA suggested that *N. neomacrospora* may be heterothallic.

Other fungi may move into the *A. tsugense* swelling once *N. neomacrospora* is established and magnify disease symptoms. For example, *Nitschikia molnarii* has been observed as a secondary invader of cankers caused by *N. neomacrospora* (Funk 1979). Many other fungi have been isolated from *A. tsugense* swellings and shoots (Baranyay 1966; Hawksworth, Wicker, and Scharpf 1977; Kuijt 1963).

1.5.3 Biology of *N. neomacrospora*

In numerous studies, *N. neomacrospora* has been isolated from the bark and wood of swollen conifer branches infected by dwarf mistletoe (Funk, Smith and Baranyay 1973; Byler and Cobb 1972). Field data suggested that *N. neomacrospora* is selective for the dwarf mistletoe swelling regardless of the host species. *Neonectria neomacrospora* has been found to be a virulent pathogen on dwarf mistletoe infected shore pine, true fir and western hemlock (Byler and Cobb 1972; Filip, Hadfield, and Schmidt 1979; Funk, Smith, and Baranyay 1973).

Research by Byler, Cobb, and Parameter (1972a, 1972b) showed that *N. neomacrospora* could also infect pine galls caused by the rust fungus *Peridermium harknessii*. Data suggested that *N. neomacrospora* could eliminate *P. harknessii* on several species of pine by killing the gall. Byler, Cobb and Parameter (1972b) found that 90 % of the pine gall rust galls treated with *N. neomacrospora* were dead within 70 weeks after inoculation. Non-galled tissues remained resistant to invasion by *N. neomacrospora*.

The ability of *N. neomacrospora* to infect dwarf mistletoe swellings and pine galls suggests that the fungus colonizes fast growing, nutritious, diseased coniferous tissue (Byler, Cobb and Parameter 1972b). Healthy coniferous tissue does not appear to be susceptible to infection. In addition to providing a nutritious environment in the swelling, dwarf mistletoe infection modifies bark moisture, which may also select for *N. neomacrospora* infection (Baranyay 1964).

It is not known which spore type of *N. neomacrospora* is the primary source of natural infection in the field, or if wounding of the host is required for *N. neomacrospora* to incite infection. Funk, Smith and Baranyay (1973) were able to establish infection on unwounded *A. tsugense* swellings using mycelium inoculum. However, a field trial conducted by Smith and Funk (1980) was unable to establish significant infection by applying a conidia and mycelium mixture onto unwounded *A. tsugense* swellings. The location of *N. neomacrospora* infection in *P. harknessii* rust galls also suggests that wounding may facilitate *N. neomacrospora* infection (Byler, Cobb and Parameter 1972b).

The location of *N. neomacrospora* within the dwarf mistletoe swelling is also poorly understood. The fungus has been isolated both from the bark and the wood of the dwarf mistletoe swelling, but it is not clear if *N. neomacrospora* invades the dwarf mistletoe endophytic system. Histological study of *N. neomacrospora* inoculated callus and germinating *A. tsugense* seeds showed that *N. neomacrospora* is able to infect *A. tsugense* tissue both inter- and intra-cellularly (Deeks, Shamoun and Punja 2002). Cell wall degradation occurred as early as 0.5 days post mycelial contact, and infection reduced the growth rate of *A. tsugense* germinated seeds.

Histological examination of rust galls inoculated with *N. neomacrospora* found both the rust and pine components of the gall killed (Byler, Cobb and Parameter 1972b). The fungal hyphae of *N. neomacrospora* tended to be localized in and around living parenchymatous cells of the gall, such as phloem, parenchyma, epithelial cells of resin ducts, and wood ray parenchyma.

1.5.4 A potential biological control agent of *Arceuthobium tsugense*.

Neonectria neomacrospora is a promising biological control agent for *A. tsugense* because it meets many of the criteria of an ideal biological control agent (Wicker and Shaw 1968). The natural distribution of *N. neomacrospora* corresponds to the distribution of the target parasite *A. tsugense* (Funk, Smith and Baranyay 1973; Byler, Cobb, and Parameter 1972a, 1972b), and the fungus has sufficient ecological amplitude to assure persistence within its host range. In addition, *N. neomacrospora* produces both asexual and sexual spores in abundance and has proven its ability to establish and spread naturally in the field (Byler, Cobb and Parameter 1972a). *Neonectria neomacrospora* is able to diminish dwarf mistletoe spread by reducing the number of healthy shoots and in some cases killing the dwarf mistletoe infection. The mechanism of *N. neomacrospora* is unique in that it attacks the dwarf mistletoe swelling and acts perennially.

The unknowns of *N. neomacrospora*, particularly the requirement of wounding for infection to occur, must be determined before this fungus is pursued further as a biological control agent for *A. tsugense*.

1.6.0 Research objectives

This study is part of a larger program to develop an inundative biological control agent for *Arceuthobium tsugense* using *Neonectria neomacrospora*. The research objectives are:

1. To select a lead isolate of *N. neomacrospora* based on growth characteristics. These include the mycelial growth over a range of temperatures, conidial germination over a range of temperatures, and conidial production on a number of solid media.
2. To determine the requirement of wounding for *N. neomacrospora* infection.
3. To measure the impact of *N. neomacrospora* infection on *A. tsugense* shoot and swelling health.

Chapters 2 and 3 describe the experimental methods used to achieve these objectives and end with an assessment of the probable efficacy of *N. neomacrospora* as an inundative biological control agent.

Chapter 2

Selection of an isolate of *Neonectria neomacrospora*

2.1.0 Introduction

Prior to commencing a field trial, it is advisable to conduct inoculation studies under controlled conditions. It is of particular importance to screen for the most virulent strain of the species under examination when developing a potential biological control agent. This idealistic approach becomes difficult, however, when working with hyperparasitic fungi.

Two attempts to develop a system that could support *A. tsugense* infections under controlled conditions were unsuccessful (data not shown). Potted hemlock seedlings inoculated with *A. tsugense* seeds under greenhouse conditions did not establish mature *A. tsugense* infections in time for this study. An alternative approach, which involved placing freshly cut hemlock branches that were naturally infected by *A. tsugense* into nutrient saturated Styrofoam blocks, did not maintain healthy *A. tsugense* infections long enough for *N. neomacrospora* to develop symptoms and signs of disease. As a consequence of the difficulties in developing *A. tsugense* experimental units under controlled conditions, it was not possible to screen *N. neomacrospora* isolates for virulence. Instead, it was decided that selection of an isolate of *N. neomacrospora* for the large field trial would be based on growth characteristics. In addition, a small field trial was conducted to develop inoculation techniques and to monitor disease development.

2.2.0 Materials and methods

Unless specified otherwise, identification of *N. neomacrospora* was based on colony and spore morphology as described in Chapter 1, Section 1.5.2 and a mycological resource monograph (Booth 1966b). Dr. Gary Samuels (pers. comm.) confirmed the identity of the *N. neomacrospora* isolate used in the small field trial. Statistical analysis was done using SigmaStat version 2.03 (SPSS Inc., Chicago, IL). All figures were drawn using SigmaPlot version 7.0 (SPSS Inc., Chicago, IL).

2.2.1 Collection of *N. neomacrospora* isolates

Diseased *A. tsugense* swellings having symptoms and signs associated with *N. neomacrospora* were collected in June and July of 2001 and January of 2002 at Horne Lake, Holt Creek, Mesachi Lake, and Muir Creek on Vancouver Island, British Columbia (BC). Previously collected and identified isolates of *N. neomacrospora* from the Pacific Forestry Centre (PFC) Culture Collection were also re-activated.

Needles and *A. tsugense* shoots were removed from the freshly collected samples in the laboratory. Sections of diseased (i.e. necrotic) bark were removed and surface sterilized for 2-minute washes in each of 95% ethanol, 10% bleach (sodium hypochlorite, 5%), and three washes of sterile, distilled water. Sterilized sections were placed on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) and incubated at room temperature (22°C) for 1-2 weeks. If fruiting bodies were present, perithecia were crushed in sterile, distilled water and streaked onto a PDA plate using a sterile loop. Sporodochia were scraped off the sample and placed onto PDA using sterile tweezers.

Small sections of resulting fungal cultures were re-plated on PDA and incubated for another 2-3 weeks at room temperature. Fungal colonies were then microscopically examined and identified. Four freshly isolated *N. neomacrospora* colonies and two *N. neomacrospora* isolates from the PFC culture collection were selected for the growth trials.

2.2.2 Selection and screening of the *N. neomacrospora* isolates

Six isolates of *N. neomacrospora* were selected for screening (Table 2.1). All isolates were collected on Vancouver Island, BC. The growth characteristics determined for each of the six isolates included the rate of mycelial growth at varying temperatures (linear growth), the percentage and rate of spore germination at varying temperatures, and the number of spores produced on different solid-based media (rice, millet, slow oats, and wheat bran).

Table 2.1. The origin of six isolates of *N. neomacrospora* selected for screening.

Isolate PFC No.	Site and Date of Collection (dd/mm/yy)	GPS (site)	Fresh Sample or Culture Collection?
PFC 4564	Spider Lake, Vancouver Island, 12/01/02	49°21'00'' lat. 124°38'00 long.	Fresh Sample
PFC 4562	Spider Lake, Vancouver Island, 12/01/02	49°21'00'' lat. 124°38'00 long.	Fresh Sample
PFC 4563	Spider Lake, Vancouver Island, 12/01/02	49°21'00'' lat. 124°38'00 long.	Fresh Sample
PFC 4561	Holt Creek, Vancouver Island, 19/07/01	49°21'00'' lat. 124°38'00 long.	Fresh Sample
PFC 2559	Holt Creek, Vancouver Island, 27/04/98	51°20'00'' lat. 117°00'00 long.	Re-activated from Culture Collection
PFC 2570	Bowser, Vancouver Island, 13/05/98	56°25'00'' lat. 129°30'00 long.	Re-activated from Culture Collection

2.2.3 Linear colony growth at varying temperature

Linear colony growth measured colony growth rate and was defined as the mean increase in radius (mm) of the colony per day. The linear colony growth was determined for the six isolates of *N. neomacrospora* at temperatures ranging from 4-30°C. The purpose of this growth trial was to establish which isolate and temperature facilitated the fastest colony growth. Mycelial PDA plugs of *N. neomacrospora* measuring 8mm in diameter were placed in the centre of fresh PDA plates and incubating at 4, 10, 15, 20, 25 and 30°C such that there were three replicates (plates) for each isolate at each temperature. The radius of each colony was measured along two points at 1, 2 and 3-

weeks after inoculation. The experiment was terminated when the hyphal margin of the fastest growing colony reached the edge of the PDA plate at 3 weeks.

2.2.4 Percentage and rate of spore germination at varying temperatures

Neonectria neomacrospora produces spores both sexually (ascospores) and asexually (conidia). Since conidia can be mass-produced in culture while ascospores cannot be produced in culture, it was decided that conidia would be used for the inoculum source in the small and large field trials. Because of this, the percentage and rate of spore germination was only measured for conidia.

The percentage and rate of conidia germination at temperatures ranging from 4-30°C was determined for the six isolates of *N. neomacrospora*. The purpose of this trial was to determine which isolate and temperature resulted in the highest percentage and the fastest rate of spore germination.

Conidia were mass-produced by inoculating 20 g of sterile rice and 20 ml of sterile, distilled water with 5 PDA plugs of mycelium in 250 ml Erylmeyer flasks. These were grown at room temperature for 7-10 days with infrequent agitation. At the end of the incubation period, 100 ml of sterile, distilled water was added to each flask. The flasks were placed on a rotary shaker at 225 rpm for 30 minutes. The contents of each flask was strained through sterile cheesecloth into a sterile beaker and transferred to multiple 50 ml centrifuge tubes. The centrifuge tubes were then centrifuged at 2500 G for 10 minutes, after which the supernatant was decanted and conidia were resuspended in 5 ml of sterile distilled water. The contents of each centrifuge tube that originated from the same isolate were combined such that there was only one centrifuge tube per isolate. Conidia were stored at 4°C and screened for bacterial contamination by plating a sub-sample of the spore solution on PDA and growing it at 30°C. Spores were used within 2 days after isolation or discarded.

Spore germination was monitored for all six isolates at 4, 10, 15, 20, 25 and 30°C. Prior to commencing the trial, the percentage of germinated spores was estimated for each isolate. If the percentage of germinated spores exceeded 10%, the spore suspension was not used in the trial. For each isolate and each temperature, 500 µl of a spore suspension containing 1×10^5 spores/ml was plated onto water agar plates. There was only

one replicate (plate) for each isolate at each temperature. At least 300 spores were counted at 2, 4, 6, 8, 10, 12 and (when necessary) 24 hours after inoculation by placing the plate directly under the microscope. Counting stopped once the percentage of germinated spores exceeded 95% or the time limit of 24 hours was reached. Counting was done in two rounds, counting three isolates at a time with staggered temperatures so that timing could be fairly accurate. In situations where the germination rate needed to be slowed for counting, plates were stored at 4°C. Spores were considered germinated if the length of the germ tube exceeded the diameter of the spore.

The percentage of germinated spores was plotted against time for each isolate at each temperature using a 3rd parameter sigmoid regression formula $Y = A/[1 + \text{Exp}((X - X_0)/B)]$, where A= maximum y-value, B=rate at which the maximum y-value was reached, Y= percentage germination at any given time (hours), X= time (hours), and X_0 =time to reach half the maximum y-value. The r^2 values for the 36 graphs varied from 0.7984 to 0.994 with a mean of 0.932 (Figure 2.1). For each isolate at each temperature, the number of hours required for 50% spore germination was estimated using the sigmoid regression formula. These data were then used as a measurement of the rate of spore germination.

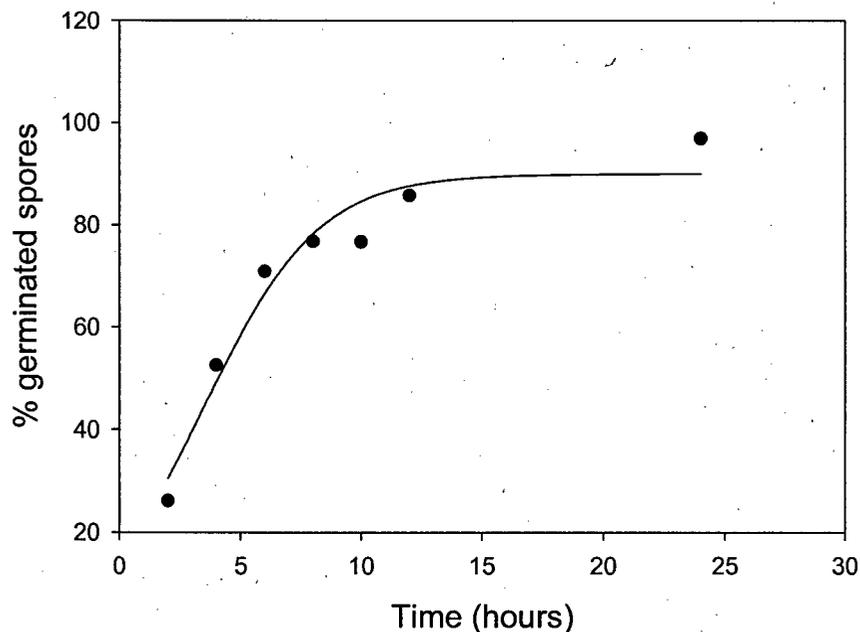


Figure 2.1. A sample graph of the percentage of germinated spores plotted against time for isolate PFC 2546 at 20°C. In this case, $r^2 = 0.95$.

2.2.5 Mass production of inoculum on different solid-based media

The spore production trial consisted of six isolates, four media types, and two replications. The purpose of this trial was to measure the number of spores produced by each isolate on different solid-based media. Although different ratios of media and water was expected to influence the number of spores produced by *N. neomacrospora*, the amount of water and media used in this trial was altered to best suit the media type.

Twenty ml of sterile distilled water was added to 20 g each of sterile rice and millet, 30 ml of sterile distilled water to 20 g of sterile slow oats, and 15 ml of sterile distilled water to 10 g of wheat bran. The media and water were mixed in 500 ml Erlenmeyer flasks and autoclaved twice to reduce contamination.

Five mycelial PDA plugs measuring 8 mm in diameter were added to each flask such that there were two replicates (flasks) of each isolate for each media type. Cultures were grown at room temperature for 12 days. Conidia were extracted using the wash technique described for the spore germination trial in section 2.2.4. Sterile, distilled water was added to the suspension such that the final total volume of the suspension was 100 ml. The number of spores in 10 μ l of suspension was then estimated using a haemocytometer.

2.2.6 Inoculation of *A. tsugense* in a small field trial

The purpose of the small field trial was to conduct a preliminary inoculation trial using three different inoculation techniques. The trial was designed to provide information on which inoculation techniques are easy to apply and permitted infection. The small field trial also served as an early warning system. Since the small field trial was situated in a slightly warmer climate and treatments were applied 1 month before the large field trial, patterns observed in the small field trial were expected to occur sooner than those observed in the large field trial.

The small field trial was located at the Pacific Forestry Centre (PFC), Victoria, BC. It consisted of eight planted hemlock trees that were inoculated with *A. tsugense*

seeds 4 years prior to treatment. At the start of the trial (July, 2002), there were a total of 21 healthy, mature *A. tsugense* infections.

Prior to treatment, notes were taken on the health of the *A. tsugense* infections and the hemlock host. The number of healthy *A. tsugense* shoots was not counted prior to treatment. The growth trials were incomplete at this time, so it was not possible to select an isolate of *N. neomacrospora* based on its growth characteristics. Instead, isolate PFC 4561 was selected for this trial because the *A. tsugense* swelling from which it was isolated was severely diseased

Two types of inoculum and three treatments were used. For the first type of inoculum, conidia were mass produced on sterile rice media for 12 days and collected using the wash method described for the spore germination trial in section 2.2.4. The spore concentration of the resulting solution was determined using a haemocytometer, and the desired concentration of spores was adjusted with sterile distilled water to a concentration of 1.0×10^7 conidia per ml. The spores were then stabilized into a granulated formulation called "Stabileze" (Quimby et al. 1999).

The Stabileze formulation was produced as follows: five grams of Waterlock™ B-204 water absorbent starch (Grain Processing Corp., Muscatine, IA, USA) was mixed with 5 ml of corn oil (Spectrum Naturals, Inc., Petaluma, CA, USA) under sterile conditions. The mixture was then heated on "high" in the microwave oven for 1 minute and allowed to cool to room temperature. Once cooled, 20 ml of conidial suspension was slowly blended into the mixture, followed by 20.0 g of confectioners' sucrose (G & W Branch, Western Sugar Co., Denver, CO, USA) and 7.0 g of Hi-Sil 233® (PPG Industries Inc., Pittsburgh, PA, USA) hydrated silica. The mixture was spread onto foil covered pans in the flow hood and allowed to air dry for 48 hours. The dried product was not passed through a series of sieves, as described in the protocol. Once dry, the product was stored in a sealed container at 4°C. For control treatments, a control Stabileze formulation was made using sterile distilled water in place of the conidial suspension.

On the day of treatment application, a small amount of the dried Stabileze formulation was added to approximately 250 ml of sterile distilled water. The resulting solution was hand shaken until the formulation was mixed thoroughly. The final spore concentration used in the small field trial was unknown. For each treatment, the Stabileze

solution was hand sprayed onto the *A. tsugense* swelling and shoots until run off. In the case of control treatments, the control Stabileze was used.

The second type of inoculum used in this trial consisted of mycelial PDA plugs. Cultures of isolate PFC 4561 were grown on PDA at room temperature for 2 weeks. Once the colonies were mature, 8 mm mycelial plugs were removed from the edge of the colonies and applied immediately to the mistletoe infected bark.

Treatments for the small field trial involved: 1) spraying unwounded *A. tsugense* swellings with Stabileze (treatment A) or control Stabileze (treatment D), 2) wounding the *A. tsugense* swelling with a cork borer and inoculating the wound with a mycelial PDA plug of *N. neomacrospora* (treatment B) or a sterile PDA plug (treatment E), then wrapping the wound with parafilm (Pichiney Plastic Packaging Inc., WI, USA), and 3) cutting off some *A. tsugense* shoots 0.5 mm from the shoot base and spraying the *A. tsugense* infection with Stabileze (treatment C) or control Stabileze (treatment F); (Table 2.2). Treatment B was applied on July 31st, 2002, and treatments A and C were applied on August 6th, 2002. A Hobo data logger (Onset, Bourne, MA) was placed in the field trial site on July 31st, 2002 to monitor temperature and humidity at 15-minute intervals for 3 weeks after inoculation (Appendices I and II).

Table 2.2. Treatment description and number of replicates for each treatment used in the small field trial.

Treatment	Treatment Description	Number of Replicates
A	Isolate PFC 4561 formulated in "Stabileze" sprayed on <i>A. tsugense</i> swellings until run off	4
B	Mycelial PDA plug of isolate PFC 4561 inserted into wound in <i>A. tsugense</i> swelling and wrapped with paraffin	4
C	Isolate PFC 4561 formulated in "Stabileze" sprayed on cut <i>A. tsugense</i> shoots until run off	4
D	Control "Stabileze" sprayed on <i>A. tsugense</i> swellings until run off	3
E	Sterile PDA plug inserted into wound in <i>A. tsugense</i> swelling and wrapped with paraffin	3
F	Control "Stabileze" sprayed on cut <i>A. tsugense</i> shoots until run off	3

Assessment occurred at 1 month after treatment and continued on a monthly basis for 1 year. Assessment measured the number of healthy and diseased *A. tsugense* shoots. Descriptive data was also collected on resinosis, cracking, and the occurrence of perithecia and sporodochia. In addition, pictures of each *A. tsugense* infection were taken at each assessment using a digital camera (Nikon Coolpix995, Chiyoda-Ku, Tokyo, Japan) and stored for future reference.

Occasionally, samples from diseased *A. tsugense* shoots and sporodochia were collected during the trial and plated onto PDA. These were grown at room temperature for 1-3 weeks and resulting fungal colonies were examined. The identity of *N. neomacrospora* was confirmed based on colony and spore morphology.

At the end of the small field trial, a sub-set of the *A. tsugense* swellings were collected and destructively examined. Re-isolation of *N. neomacrospora* from the necrotic bark and wood of the *A. tsugense* swellings was attempted. No re-isolation from *A. tsugense* shoots was done at this time. Re-isolation involved cutting small sections from the *A. tsugense* swelling and surface sterilizing them for 2 minutes in each of 10% bleach, 95% ethanol, and three washes of sterile distilled water. The sterile sections were then plated onto PDA, grown at 20°C and monitored on a weekly basis for 3 weeks. Fungal colonies were sub-cultured and *N. neomacrospora* was confirmed based on morphological and spore characteristics.

2.3.0 Results

2.3.1 Selection and screening of the *N. neomacrospora* isolates

2.3.1.1 Linear colony growth at varying temperatures

At week 3, isolate PFC 2559 grew marginally faster at 10, 15 and 20°C. All isolates grew best at temperatures ranging from 15°C to 20°C and peaked at 20°C (Figure 2.2). The colony radius appeared to be most severely inhibited at 30°C (mean radius at 21 days= 7.2 mm). The greatest variability in linear colony growth amongst isolates occurred at 25°C.

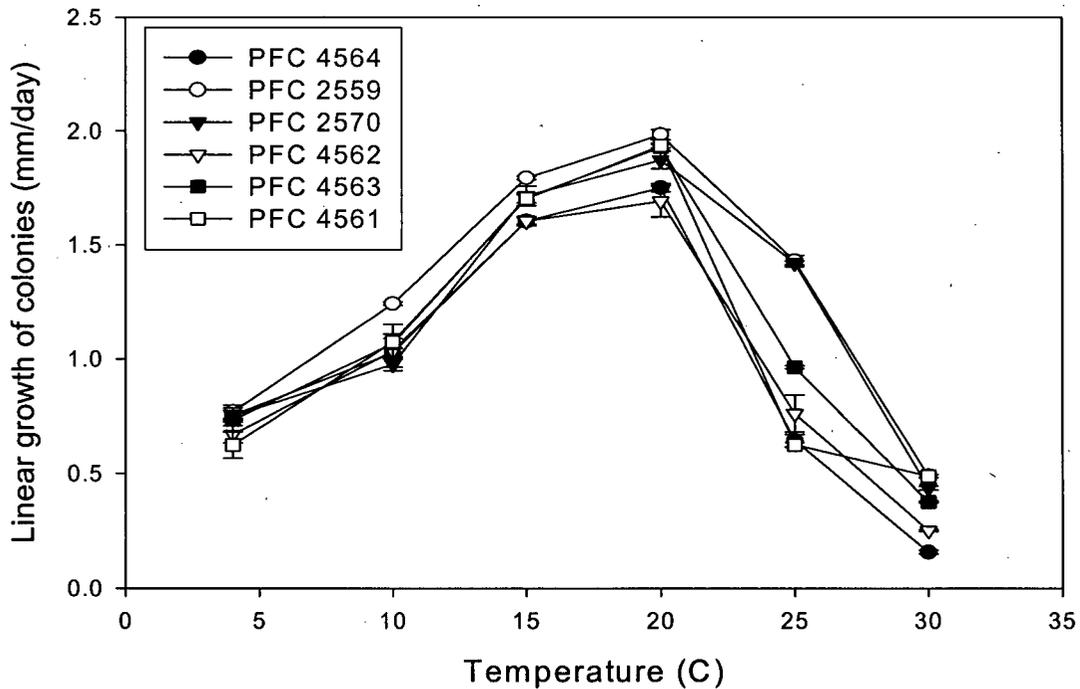


Figure 2.2. Linear colony growth (mm/day) for six isolates of *N. neomacrospora* at temperatures ranging from 4 to 30°C. Bars measure standard error of the mean.

When the colony radius at 20°C was plotted over time, the relationship for isolates PFC 4564, PFC 2559, and PFC 2570 was slightly curved (Figure 2.3). The rate of colony growth during the second week was marginally faster than during the third week. The remaining isolates showed more linear relationships and consistent rates of colony growth.

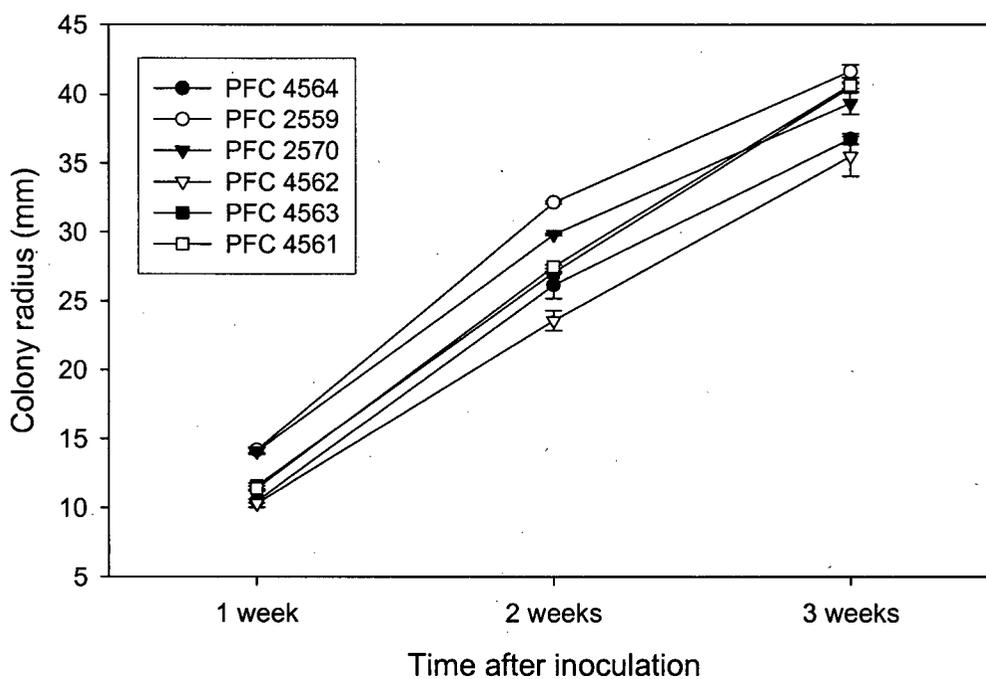


Figure 2.3. Colony radius (mm) across time for six isolates of *N. neomacrospora* at 20°C. Bars measure standard error of the means.

2.3.1.2 Percentage and rate of spore germination at varying temperatures

At 24 hours after plating, at least 78% of the spores for each isolate had germinated across a broad temperature range (Figure 2.4). Lower and higher temperatures did not appear to inhibit spore germination for the six isolates of *N. neomacrospora*.

Isolate PFC 4562 (96-97%) had the smallest range and the second highest percentage of germinated spores across all temperatures when compared to the other isolates. At 20°C, isolate PFC 2559 had the lowest percentage of germinated spores (82.5%) when compared to the other isolates. After completion of the germination trial, spore germination was monitored at 35°C, but no significant increase in the percentage of germinated spores was observed 24 hours after inoculation and counting was terminated.

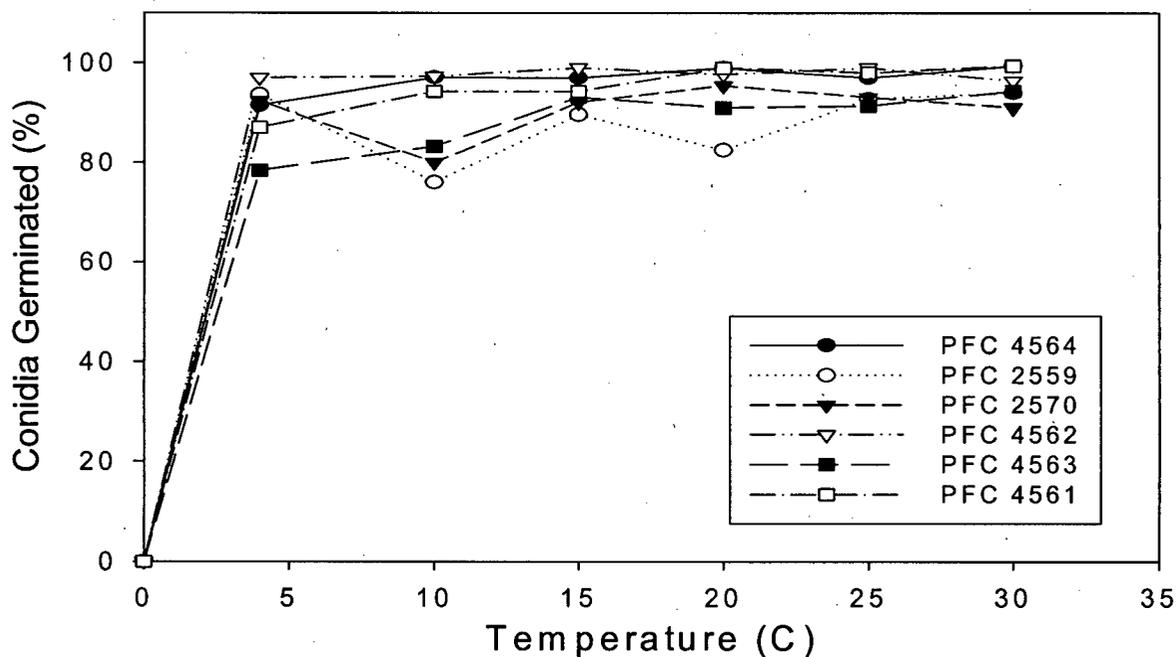


Figure 2.4. Percent conidial germination for six isolates of *N. neomacrospora* 24 hours after inoculation at temperatures ranging from 4°C to 35°C.

The estimated time required to reach 50% germination was highly variable among isolates (Figure 2.5). Based on calculations, conidia from isolate PFC 2570 took less time to reach 50% germination than the other isolates at all temperatures except for 30°C. At 20°C, isolate PFC 2570 reached 50% germination in approximately 1.4 hours. The rate of spore germination for all isolates was slowest at 4°C and 10°C. The rate of spore germination was fastest at 30°C (mean = 3.0 hours for 50% germination).

It is important to note that, although spores were capable of germinating at relatively low and high temperatures, the health of germinated spores and early mycelial development was poorer at temperature extremes. This was especially true for spores that germinated at 30°C or higher.

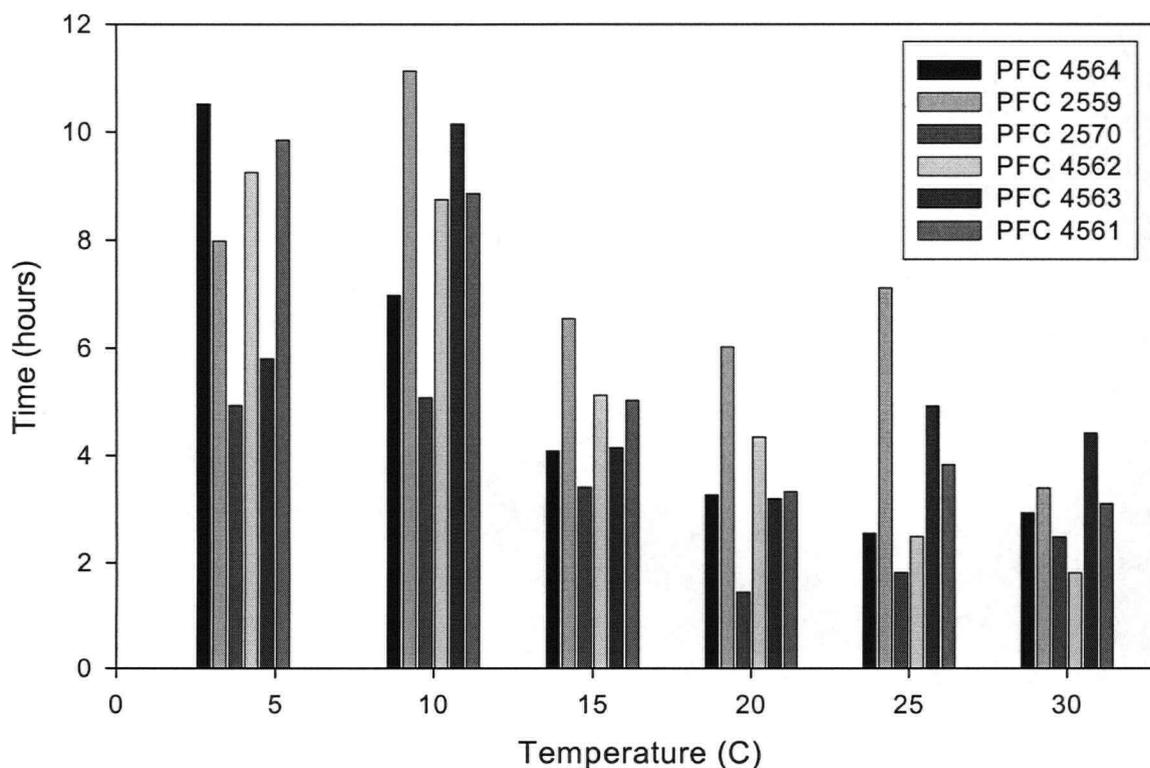


Figure 2.5. Number of hours for 50% spore germination for six isolates of *Neonectria neomacrospora* at temperatures ranging from 4°C to 30°C.

2.3.1.3 Mass production of inoculum on different solid-based media

Both macroconidia and microconidia were produced in culture. Isolate PFC 2559 produced the greatest number of spores (74 spores per μl) when compared to the other isolates (Figure 2.6). Isolate PFC 4654 (11 spores per μl) produced the fewest number of spores. The number of spores produced by isolates PFC 2570, PFC 4562, PFC 4563, and PFC 4561 ranged from 16-34 spores per μl .

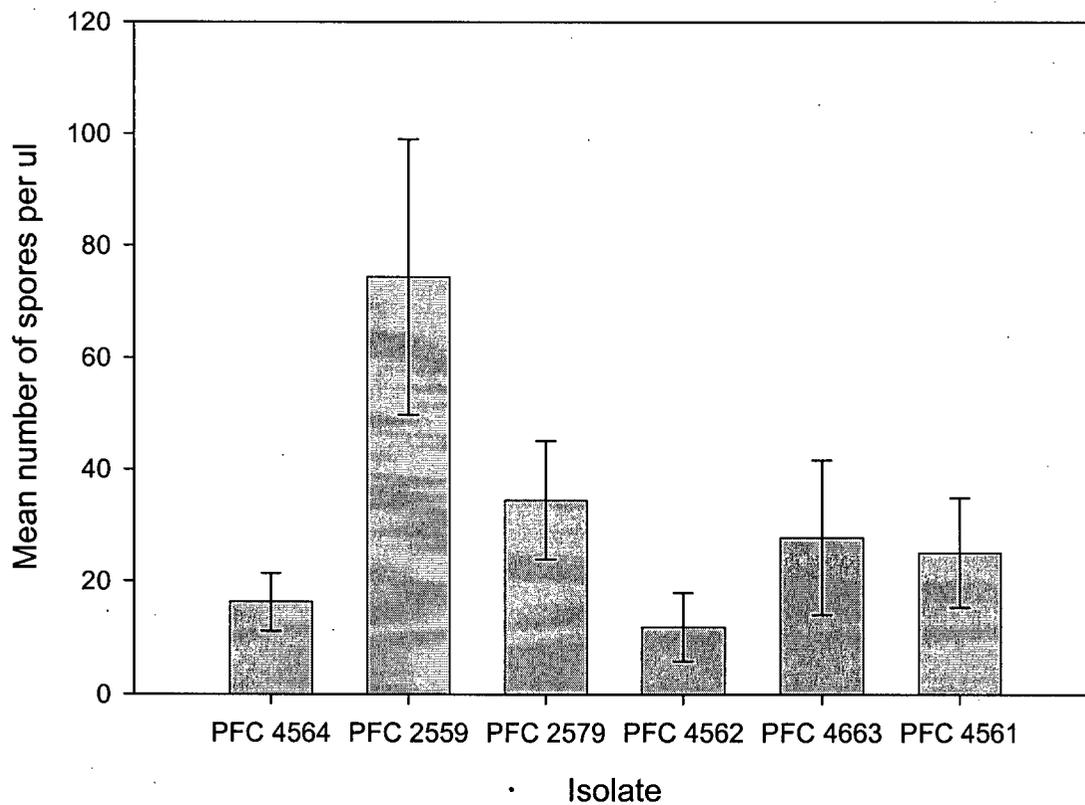


Figure 2.6. Mean number of spores produced by six isolates of *N. neomacrospora* averaged across four media types (brown rice, millet, wheat bran and slow oats) at room temperature (22°C). Bars measure standard error of the mean.

When comparing the four media types, rice supported the greatest production of spores (51 spores per μl); (Figure 2.7). This was followed by millet (40 spores per μl) and wheat bran (30 spores per μl) produced an intermediate numbers of spores. Slow oats produced the least number of spores (5 spores per μl). There was also a range in spore size and consistency across the media types, where different media tended to produce larger, more uniform spores (data not shown).

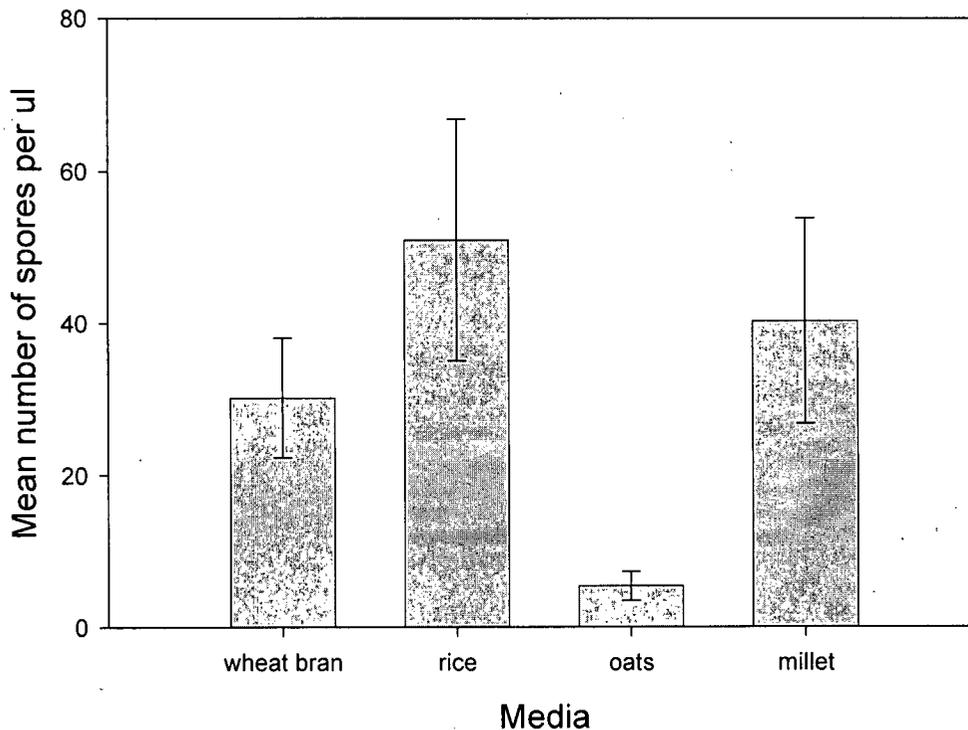


Figure 2.7. Mean number of spores produced on four media types averaged across six isolates of *N. neomacrospora* at room temperature (22°C). Bars measure standard error of the mean.

Two-way repeated measures analysis of variance on ranks detected a significant difference in spore production among the different media types ($P=0.005$). Tukey's test found rice, wheat bran, and millet each had significantly more spores than slow oats ($P=0.005$, $P=0.009$, $P=0.010$, respectively). Statistical analysis did not detect a significant

difference between isolate types or a significant interaction between media and isolate types.

2.3.2 Inoculation of *A. tsugense* in the small field trial

The temperature and relative humidity for the small field trial is shown in Appendices I and II. Treatment B and its respective control were applied in the late afternoon on July 31st, 2002, and the highest temperature was 24°C at 4:48 pm. The lowest relative humidity was 38% at 5: 03 pm. On the day of inoculation for treatments A and C and their respective controls (August 6th, 2002), the highest temperature was 23°C at 12:48 pm. The lowest relative humidity was 50% at 4:18 pm.

Trends in the number of healthy *A. tsugense* shoots were apparent (Figure 2.8). Since the number of healthy *A. tsugense* shoots was not recorded before treatment, data from the first assessment at 1 month is also included in Figure 2.8.

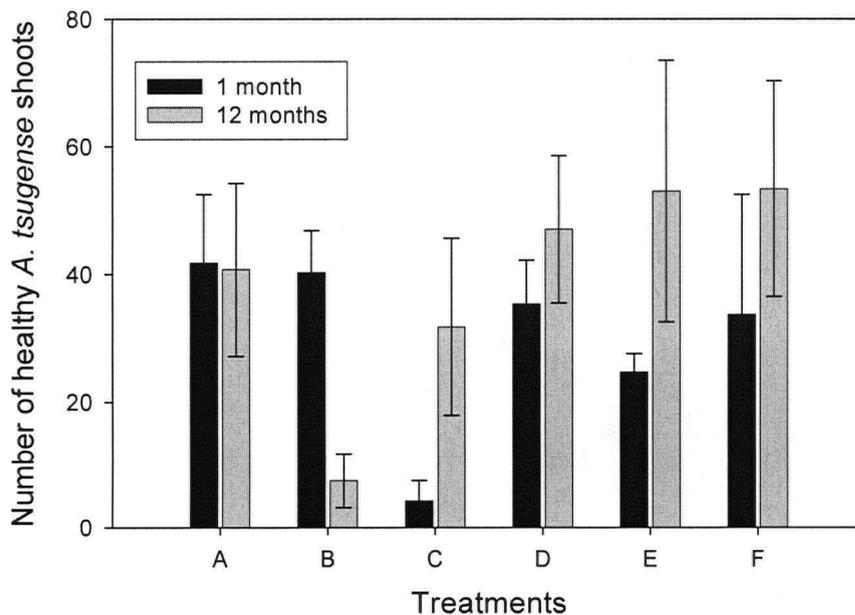


Figure 2.8. Mean number of healthy *A. tsugense* shoots observed at 1 and 12 months after treatment. Bars measure standard error of the mean.

The mean number of shoots for the unwounded, Stabileze inoculated treatment (A) and its respective control (D) did not vary at 1 and 12 months after treatment. The cut shoots, Stabileze inoculated treatment (C) and its respective control (F) appeared to recover at 12 months from treatment. However, the wound, mycelial plug inoculated treatment (B) had a large reduction in the number of healthy dwarf mistletoe shoots at 12 months when compared to its respective control (E). There were not enough replicates used in the small field trial to detect statistically significant differences in the number of healthy *A. tsugense* shoots between treatments.

Confirmation that *N. neomacrospora* had colonized *A. tsugense* swellings in the small field trial was based on the occurrence of sporodochia (Table 2.3). An attempt to re-isolate *N. neomacrospora* from a few of the *A. tsugense* swellings was also made. Based on these data, treatment B appeared to have the highest number of colonized *A. tsugense* swellings (100%) when compared the other treatments. One year after treatment, sporodochia occurred as far as 40 mm away from the site of inoculation on an *A. tsugense* swelling from treatment B.

Dr. Gary Samuels confirmed the identity of two isolates of *N. neomacrospora* isolated from dead *A. tsugense* shoots and basal cups (pers. comm.).

Table 2.3. Sporodochia and successful re-isolation of *N. neomacrospora* (N. n.) for each treatment from the small field trial. The proportion of *A. tsugense* swellings colonized by *N. neomacrospora* was based on the occurrence of sporodochia only.

Treatment	Number of Replications	Sporodochia Observed	Re-isolation of N. n.	Proportion of <i>A. tsugense</i> swellings colonized by N. n.
A	4	2/4	2/4	50%
B	4	4/4	4/4	100%
C	4	1/4	0/4	25%
D	3	1/3	0/0	33%
E	3	2/3	0/1	67%
F	3	1/3	0/0	33%

2.4.0 Discussion

2.4.1 Growth characteristics of *N. neomacrospora*

It was assumed that the rate of colony growth of *N. neomacrospora* on PDA approximates *N. neomacrospora* vigour in the field under similar growth conditions. The linear colony growth of the six *N. neomacrospora* isolates was fairly uniform for temperatures at and below 20°C (Figure 2.2). All six isolates grew best at temperatures ranging from 15°C to 20°C and had fastest linear colony growth at 20°C. These findings corroborate previous studies in which 6 of 7 isolates of *N. neomacrospora* (as *Nectria macrospora*) were shown to grow best at 21°C (Ouellette, 1973). Isolate PFC 2559 grew marginally faster than the other isolates at 10°C, 15°C, and 20°C, suggesting that it was the best candidate for the field trial in terms of colonization.

The percentage and rate of spore germination are important measurements because they provide an estimate of infection potential, particularly the minimum duration of the infection window. The percentage of spores germinated at 24 hours was fairly uniform for the six isolates at all temperatures (Figure 2.4). Lower and higher temperatures did not appear to reduce the total percentage of germinated spores at 24 hours. Based on calculations, the conidia from isolate PFC 2570 reached 50% germination faster than any other isolate at 4, 10, 15, 20 and 25°C (Figure 2.5).

The production of spores on four different media types was measured to determine which isolate and solid-based media resulted in the greatest mass production of inoculum. Of the six isolates, isolate PFC 2559 produced the greatest number of spores averaged across the four solid-based media, but this was not significantly greater than the other isolates. Rice produced significantly more spores than the other solid-based media types, and slow oats produced the fewest spores. Statistical analysis failed to detect a significant interaction between media and isolates. Based on these findings, it was decided that growing isolate PFC 2559 on rice media would result in the greatest amount of conidia production.

When taking all three growth trials into account, isolate PFC 2559 was selected for the large field trial. Isolate PFC 2559 had the fastest colony growth and produced the greatest number of spores. These characteristics will make it easy to mass-produce inoculum for this isolate. The rate and percentage of spore germination for isolate PFC

2559 was marginally smaller than that observed for isolate PFC 2570, but was assumed to be sufficient for use in the field trial.

2.4.2 Small field trial.

At 12 months, the wounded, plug inoculated treatment (B) resulted in a greater reduction in the number of healthy *A. tsugense* shoots than the other inoculated treatments (A and C); (Figure 2.8). This suggests that applying mycelial inoculum to wounded *A. tsugense* swellings has a greater impact on *A. tsugense* shoot vigour than applying inoculum to cut *A. tsugense* shoots or unwounded *A. tsugense* swellings. The number of healthy *A. tsugense* shoots observed for the unwounded, inoculated treatment (A) did not vary significantly during the field trial. In the case of the cut shoots, inoculated treatment (C), the *A. tsugense* infections had mostly recovered from treatment at 12 months.

Sporodochia first appeared October 15th, 2002 (10 weeks after treatment) and were most prominent at 3.5, 4.5 and 7.5 months after treatment. Of the inoculated treatments, the wounded, plug inoculated treatment (B) had the highest occurrence of sporodochia (100% of *A. tsugense* swellings); (Table 2.3). The occurrence of sporodochia for the remaining inoculated treatments and uninoculated controls ranged from 33-50%.

The occurrence of sporodochia for the uninoculated treatments (D, E, and F) was unexpected. There were no known nearby natural *N. neomacrospora* inoculum sources at the field trial site. It is unclear if *A. tsugense* infections in the uninoculated treatments were contamination by the inoculated treatments, either during the inoculation process or by secondary infection. Secondary infection via sporodochia is less likely, as sporodochia first appeared on control treatments only 2 weeks after the first appearance of sporodochia on inoculated treatments. The treated *A. tsugense* swellings were within close proximity of one another (0.5- 5.0 m), allowing for easy spread of inoculum.

The maximal distance between sporodochia and the inoculation site observed in the small field trial at 1 year was 40mm. Funk, Smith and Baranyay (1973) observed a similar maximal distance between sporodochia and the inoculation site (39 mm/year). These data suggest that *N. neomacrospora* is successfully moving through the *A. tsugense* swelling. In addition, the rate of movement observed in both trials approximates the rate

of *A. tsugense* spread in the hemlock host (Funk, Smith and Baranyay 1973). These data indicate that *N. neomacrospora* may be able to compete with the growth rate of *A. tsugense* and thereby have a long-term impact on *A. tsugense* vigour.

Disease symptoms were greatest for the wounded, plug inoculated treatment (B); (Figure 2.9). All four *A. tsugense* swellings from the wounded, plug inoculated treatment (B) were completely girdled, killing the lateral *A. tsugense* swelling and host branch. The earliest experimental unit to become girdled occurred at 8 months after treatment. All the *A. tsugense* shoots beyond the point of girdling died quickly after girdling occurred. In many cases, “mummified” dark brown and black shoots remained attached to the swelling. Several attempts to re-isolate *N. neomacrospora* from these diseased and dead shoots were made. In two cases, the fungus was successfully re-isolated from the dead *A. tsugense* shoots and cups, suggesting that *N. neomacrospora* may be able to move into diseased and dead *A. tsugense* shoots as a saprophyte.

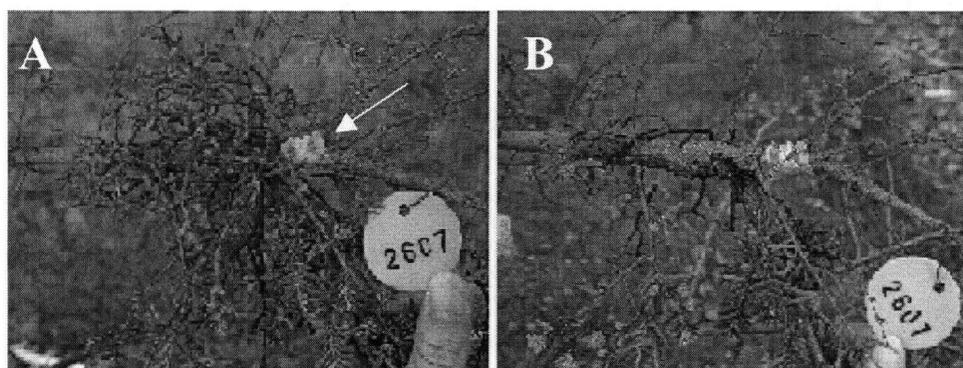


Figure 2.9. Symptoms of *A. tsugense* infected with a mycelial plug of *N. neomacrospora* into a wound at 1 month (A) and 9 months (B). The arrow in (A) points to the site of inoculation. Note the diseased, dead *A. tsugense* shoots and girdling of the swelling at 9 months (B).

One of the two girdled *A. tsugense* swellings from the unwounded, inoculated treatment (A) was situated 0.5 m directly below an *A. tsugense* infection from a wounded, plug inoculated treatment (B). Sporodochia were observed on the wounded, plug inoculated infection (B) as early as January 2002, and numerous diseased and dead *A. tsugense* shoots fell from the higher wounded, plug inoculated infection (B) onto the

lower unwounded, inoculated infection (A) during the field trial. Because of this, it is not known if infection of the unwounded, inoculated infection (A) resulted from the treatment itself or by natural infection. In either event, the *A. tsugense* swelling was not mechanically wounded.

Results from the small field trial suggest that the wounded, plug inoculated treatment (B) had the greatest impact on *A. tsugense* shoot health and number, and also caused the greatest amount of girdling of the *A. tsugense* swelling. In addition, sporodochia were most commonly produced by the wounded, plug inoculated treatment (B). The unwounded, inoculated treatment (A) and cut shoots, inoculated treatment (C) had relatively small impacts on *A. tsugense* shoot and swelling vigour when compared to the wounded, plug inoculated treatment (B). Observations from the small field trial also confirmed that, once established, *N. neomacrospora* is able to reduce *A. tsugense* shoot and swelling vigour (Figure 2.8, 2.9).

Chapter 3

Inoculation of *Arceuthobium tsugense* in a large field trial

3.1.0 Introduction

The infection process of *N. neomacrospora* is not well understood. This species has two separate life stages and produces spores both sexually (ascospores) and asexually (conidia). It is not known which of these spore types is the primary, natural source of inoculum in the field. Conidia can be easily mass-produced in the laboratory, but a technique to mass-produce ascospores in the laboratory has not yet been developed. Both conidial and perithecial stages occur in the field.

Smith and Funk (1980) used a mycelium and conidia mixture suspended in water to inoculate unwounded *A. tsugense* swellings. Only 8 of the 20 the treated and 3 of the 20 control *A. tsugense* swellings became colonized by *N. neomacrospora*. It was not known if the form of inoculum or the inoculation process itself was responsible for the low infection, and it was suggested that an improved inoculum technique must be developed. No published trials have used ascospores as an inoculum source.

With these considerations in mind, it was decided that conidia would be incorporated into a Stabileze formulation for the large field trial. The Stabileze formulation was selected because it is simple, inexpensive, and can improve survival of fungal pathogens (Quimby et al. 1999). Other studies with different species of fungi have found that formulation can extend the period of time in which the fungus remains viable, reduce the dew period requirements for infection, and reduce the spray volume by 100-fold (Boyette 1994). Preliminary work with *N. neomacrospora* conidia formulated in Stabileze showed that the fungus was able to germinate from dry granules on PDA within 48 hours (data not shown). This is the first experiment to use *N. neomacrospora* spores formulated in Stabileze.

There is conflicting evidence about whether wounding of the *A. tsugense* swelling or shoots is required for infection by *N. neomacrospora* to occur. The low infection observed for a trial in which mycelium and conidia mixed in water were applied to unwounded *A. tsugense* infections suggests that *N. neomacrospora* can not penetrate

unwounded *A. tsugense* swellings (Smith and Funk 1980). In contrast, another trial that applied mycelial inoculum over basal cups and young shoots showed successful inoculation of unwounded *A. tsugense* swellings (Funk, Smith and Baranyay 1973). Further research is required to clarify these findings.

Measurement of symptom development, with particular emphasis on the rate of *A. tsugense* shoot reduction and the impact on *A. tsugense* swelling health also deserve more study. Only one previous inoculation trial has measured the impact of *N. neomacrospora* infection on the number of *A. tsugense* shoots (Smith and Funk 1980), but data from this study was only taken at the end of the trial and was not measured on a monthly basis.

Another trial that monitored natural *N. neomacrospora* infection on dwarf mistletoe attributed a 30% reduction in the number of *A. tsugense* shoots to infection (Funk, Smith and Baranyay 1973). These data, however, applied to different aged *N. neomacrospora* infections and also failed to measure the impact of infection on a monthly basis.

The purpose of the large field trial was to determine if wounding of the *A. tsugense* swelling or shoots is required for *N. neomacrospora* infection. In addition, the large field trial measured the impact of *N. neomacrospora* infection on *A. tsugense* shoot production and bark necrosis of the *A. tsugense* swelling.

3.2.0 Materials and methods

In similar fashion to the small field trial, the identification of *N. neomacrospora* was based on colony and spore morphology as described in Chapter 1, Section 1.5.2 and a mycological resource monograph (Booth 1966b). The mycologist Dr. Gary Samuels confirmed the identity of the *N. neomacrospora* isolate used in the large field trial (pers. comm.).

3.2.1 Stabileze formulation

Mass inoculum of *N. neomacrospora* isolate PFC 2559 was produced on sterile rice media and harvested at 12 days using the wash method previously described for the spore germination trial in section 2.2.4. The spores were then incorporated into Stabileze using the technique described for the small field trial in section 2.2.6. For control

treatments, a control Stabileze formulation was made using sterile distilled water in place of the conidial suspension.

On the day of the treatment application, 5.0 g of the dried formulation was added to 500 ml of sterile, distilled water. The solution was alternatively hand shaken and stirred on a rotary shaker for 30 minutes, or until the formulation was mixed thoroughly. A total of three batches of 500 ml of Stabileze solution and three batches of 500 ml of the control Stabileze solution were made. The final concentration of spores was approximately 54000 conidia per ml. For each treatment and control, the Stabileze solution was hand sprayed onto the *A. tsugense* swelling and shoots until run off.

Prior to commencing the field trial, germination experiments using formulated conidia were done in the laboratory to ensure that *N. neomacrospora* spores remained viable in the Stabileze formulation. These preliminary trials showed successful germination and colony development of *N. neomacrospora* on PDA for Stabileze in both dry and water suspended forms. The exact spore number and percentage germination could not be determined because spores were hidden in the Stabileze aggregates. Other laboratory work failed to detect self-inhibited spore germination at the spore concentration used in the large field trial.

3.2.2 Site description

The field trial site was established in a mixed conifer stand heavily infested by *A. tsugense* near Parksville, Vancouver Island, BC. The field site was distributed across approximately 2 hectares in a Coastal Douglas-fir Zone following the biogeoclimatic ecosystem classification system employed by the British Columbia Ministry of Forests (1992). Hemlock was the primary tree species and ranged in age from 5 to 40 years. Other common tree species included Arbutus (*Arbutus menziesii* Pursh.), white pine, Douglas fir and occasionally cedar. The site had been thinned irregularly and had patches of open areas. Salal (*Gaultheria shallon* Pursh.), some *Rubus* species, sword fern (*Polystichum munitum* (Kaulf.) Presl) and bracken fern (*Pteridium aquilinum* (L.) Kuhn) were common. The site was heavily infested with *Arceuthobium tsugense*, with almost all hemlock having at least one and as many as 15 or more *A. tsugense* infections.

Naturally occurring inoculum of *Neonectria neomacrospora* and the *A. tsugense* shoot pathogen *Colletotrichum gloeosporioides* were present at the site and colonizing *A. tsugense* infections. A Hobo data logger (Onset, Bourne, MA) was installed at the site on the day of inoculation to record temperature and relative humidity (%). The Hobo data logger collected data at 30-minute intervals for 3 months following treatment.

3.2.3 Experimental design

Selection of *A. tsugense* experimental units was based on the following criteria: 1) the *A. tsugense* shoots and swelling were disease free, with only a single *A. tsugense* infection on the host branch, 2) the host hemlock branch was vigorous, and 3) the infection was accessible and easy to monitor. Both male and female *A. tsugense* infections of various sizes were used in this trial.

A total of 237 *A. tsugense* infections were selected, tagged, and randomly assigned one of seven treatments (Table 3.1). There were 35 replicates of treatments A, B, C, J, K, and L, and 27 replicates of treatment N. Treatments were assigned randomly, regardless of the sex, size, or location of the *A. tsugense* infection. In some cases, there was more than one *A. tsugense* infection on the same tree; it was assumed that all host trees would act similarly in the field and these *A. tsugense* were also randomly assigned treatments. The number of young hemlock trees used for each treatment was as follows: treatment A (26), B (34), C (29), J (30), K (28), L (29), and N (27). There was some overlap where different treatments occurred on the same tree.

Table 3.1. Treatment description and number of replicates used in the field trial designed to assess *N. neomacrospora* as a biological control agent for *A. tsugense*.

Treatment	Treatment Description	Number of Replicates
A	Isolate PFC 2559 formulated in "Stabileze" sprayed on <i>A. tsugense</i> infections to run off	35
B	Isolate PFC 2559 formulated in "Stabileze" sprayed on cut shoots of <i>A. tsugense</i> to run off	35
C	Isolate PFC 2559 formulated in "Stabileze" sprayed on wounded swelling of <i>A. tsugense</i> infections to run off	35
J	Control "Stabileze" sprayed on <i>A. tsugense</i> infections to run off	35
K	Control "Stabileze" sprayed on cut shoots of <i>A. tsugense</i> to run off	35
L	Control "Stabileze" sprayed on wounded swelling of <i>A. tsugense</i> infections to run off	35
N	The <i>A. tsugense</i> infection was not treated	27

The purpose of treatments A and J was to determine if applying formulated conidia of *N. neomacrospora* onto unwounded *A. tsugense* is sufficient to permit infection. Treatment involved spraying unwounded *A. tsugense* with Stabileze (treatment A) or control Stabileze (treatment J) until run off.

The purpose of treatments B and K was to determine if applying formulated conidia of *N. neomacrospora* onto cut *A. tsugense* shoots facilitates infection. Data from these treatments will ascertain if *N. neomacrospora* is able to enter the *A. tsugense* swelling by colonizing wounded *A. tsugense* shoots. For these treatments, all *A. tsugense* shoots were cut 0.5 mm from the base using scissors sterilized in 95% ethanol. The entire infection was then sprayed with Stabileze (treatment B) or control Stabileze (treatment K) until run off. Treatment A served as the unwounded, inoculated control and treatment K as the cut shoots, uninoculated control (=respective control).

The purpose of treatments C and L was to determine if wounding *A. tsugense* swellings facilitates infection. Treatment involved applying numerous incisions that penetrated beyond the cambium along the *A. tsugense* swelling using a razor blade sterilized in 95% ethanol. The incisions were made perpendicular to the host branch and did not girdle the swelling. The *A. tsugense* swelling and shoots were then sprayed with

Stabileze (treatment C) or control Stabileze (treatment L) until run off. Treatment A served as the unwounded, inoculated control, and treatment L as the wounded, uninoculated control (=respective control).

Treatment N received no treatment and was compared to treatment J to determine if spraying *A. tsugense* swellings and shoots with control Stabileze increased natural infection via naturally occurring *N. neomacrospora* inoculum. Treatment N was also a measurement of the proportion of experimental units that appeared healthy during the selection process but were naturally infected by *N. neomacrospora* either before or during the trial.

The Stabileze and control Stabileze formulations used in the field trial were grown on PDA directly following treatment application. This was done to confirm that the spores used in the trial were viable. The plates were incubated at 20°C for 1-2 weeks.

3.2.4 Pre-treatment assessment

Assessment of each experimental unit was done prior to treatment application on August 21st and 22nd, 2002. Pre-treatment assessment measured number of healthy *A. tsugense* shoots and the number of *A. tsugense* buds (shoots measuring less than 5 mm in height).

3.2.5 Treatment application dates

All treatments were applied on August 29th, 2002. The weather during the day of treatment was sunny and warm with temperatures ranging from 19 to 24°C during the day down to 7°C at night. The highest temperature was 24°C and occurred at 1:35 pm. Relative humidity ranged from 52 to 80% during the day with a minimal humidity of 53% at 3:35 pm. The maximal relative humidity that night occurred at 11:35 pm and measured 91%. The following week had moderate temperatures ranging from 7 to 23°C, and relative humidity ranging from 40-100% (Appendices III and IV). The trial was intended to continue for 1 year.

3.2.6 Treatment assessment

Assessment after treatment measured the number of healthy *A. tsugense* shoots and the number of *A. tsugense* buds. The presence of sporodochia, perithecia, resinosis and cracking of the bark were also noted. All treatments were assessed at 2 weeks and 1, 2, 3.5, 5, 6, and 9 months after treatment application. Also, along with each assessment, a photographic record of a few representative *A. tsugense* infections from each treatment was taken using a digital camera (Nikon Coolpix995, Chiyoda-Ku, Tokyo, Japan) and saved for future reference.

3.2.7 Sampling at 10 months

Because of the high occurrence of sporodochia observed in the field and concerns about secondary infection of treatments, the large field trial was terminated early at 9 months after treatment. At 10 months, a total of 20 experimental units were randomly collected from each of treatments A, B, C, K, and L, 21 samples from treatment J, and 11 samples from treatment N (Table 3.2). These units were studied more thoroughly in the laboratory, and measurements of bark necrosis and the occurrence of sporodochia were taken. An attempt to re-isolate *N. neomacrospora* from *A. tsugense* swellings was also made.

Table 3.2. The number of experimental units collected from the trial 10 months after treatment. Note that the total number of experimental units ("Total") has taken into account *A. tsugense* infections that were lost during the trial (Table 3.4).

Treatment	A	B	C	J	K	L	N
Collected	20	20	20	21	20	20	11
Remaining	13	15	13	14	14	15	16
Total	33	35	33	35	33	35	27

The extent of bark necrosis for each *A. tsugense* swelling was based on the circumference of bark necrosis at the widest point divided by the total circumference of the swelling at that point. The circumference was used to measure the extent of bark necrosis rather than a longitudinal measurement because it gives an estimate of how close

the swelling is to being girdled. Girdling of the swellings kills the distal part of the *A. tsugense* infection and therefore has a significant impact on *A. tsugense* shoot and seed production.

The occurrence of sporodochia on destructively sampled *A. tsugense* swellings at the end of the field trial was treated as non-cumulative data. Microscopic observations of the spore-producing structures confirmed the identity of sporodochia. Data measuring the occurrence of sporodochia during the large field trial were kept separate and were not statistically analyzed. Field data on sporodochia were not analyzed because sporodochia may have been missed or falsely identified during assessment.

To re-isolate *N. neomacrospora*, 10 small sections of bark and wood were cut from the *A. tsugense* swelling and surface sterilized for 2 minutes in each of 10% bleach, 95% ethanol, and three washes of sterile distilled water. When cutting out sections from the *A. tsugense* swelling, preference was given to the margins of necrotic bark and nearby wood regions. In cases where necrotic bark did not occur, sections were taken at random from the bark and wood of the *A. tsugense* swelling. The sterile sections were plated onto PDA, incubated at 20°C and monitored on a weekly basis for 3 weeks. Fungal colonies were sub-cultured and *N. neomacrospora* was confirmed based on culture and spore characteristics.

3.2.8 Statistical analysis

All statistical analyses were performed using SigmaStat version 2.03 (SPSS Inc., Chicago, IL) and graphs were drawn using SigmaPlot version 7.0 (SPSS Inc., Chicago, IL). In situations where multiple comparisons between treatments were made, the alpha level of the test was not altered and remained $\alpha=0.05$. This is because comparisons of interest were determined prior to experimentation; these include treatments A vs. C, A vs. J, A vs. B, B vs C, B vs. K, and C vs. L.

3.2.8.1 Statistical analysis of the impact of wounding on infection

The extent of bark necrosis, the occurrence of sporodochia and successful re-isolation of *N. neomacrospora* for each treatment were used to address the first objective. The extent of bark necrosis, re-isolation and the non-cumulative occurrence of

sporodochia at 10 months were based only on the destructively sampled *A. tsugense* swellings.

Data measuring the extent of bark necrosis did not meet the assumptions of analysis of variance and transformation using arcsin-square-root (x) did not remedy this problem. Analysis of variance was therefore done using a two-way analysis of variance on ranks for treatments A, C, J, and L and for treatments A, B, J, and K. Treatments were grouped in this manner for practical purposes; there was more interest in determining where, if any, interactions between treatment types occurred in addition to the significance of differences. By separating treatments, it was possible to narrow the location of interaction. The Tukey test was used for pairwise comparisons.

Chi-Square analysis was used to test if significant differences occurred between treatments for the non-cumulative occurrence of sporodochia and re-isolation of *N. neomacrospora*. The Fisher Exact test was used to compare differences between two treatments. Data collected during the field trial on the occurrence of sporodochia were not statistically analyzed.

Comparison of successful re-isolation of *N. neomacrospora* versus the occurrence of sporodochia on the destructively sampled *A. tsugense* swellings was done using Chi-Square analysis. The relationship between different classes of bark necrosis and the percentage of *A. tsugense* swellings with confirmed *N. neomacrospora* infection was analyzed using linear regression.

3.2.8.2 Statistical analysis of the impact of infection on *A. tsugense* vigour

The impact of *N. neomacrospora* infection on *A. tsugense* vigour took into account the number of healthy *A. tsugense* shoots, proportion of *A. tsugense* infections with zero shoots, and the extent of bark necrosis and girdling of the *A. tsugense* swelling. All data measuring the impact of infection on *A. tsugense* vigour were based on destructively examined samples only. The *A. tsugense* infections from all treatments were re-grouped into two groups: those that were confirmed to be colonized by *N. neomacrospora* (=confirmed group), and those that were not confirmed to be colonized by *N. neomacrospora* (=unconfirmed group); (Table 3.3). Confirmation that infection by

N. neomacrospora had occurred was based on successful re-isolation of the fungus and/or confirmation of sporodochia at the end of the large field trial.

Table 3.3. Break down of the number of *A. tsugense* swellings with confirmed and unconfirmed *N. neomacrospora* infection for each treatment.

	Treatments						
	A	B	C	J	K	L	N
No. confirmed	11	14	18	6	4	7	2
No. unconfirmed	9	6	2	15	16	13	9

The assumptions of a t-test were not met, and so a Mann-Whitney Rank Sum test was used to statistically analyze the number of healthy *A. tsugense* shoots and the extent of bark necrosis for the confirmed and unconfirmed groups. The proportion of *A. tsugense* infections with zero shoots and the proportion of girdled *A. tsugense* swellings were statistically analyzed using Chi-square analysis followed by the Fisher exact test. In the case of the number of healthy *A. tsugense* shoots and the proportion of *A. tsugense* swelling with zero shoots, treatments B and K were removed from data analysis because the treatment process itself (i.e., cutting shoots) may have influenced the variable of interest.

3.3.0 Results

3.3.1 Stabileze formulation

The field trial used the formulation technique described by Quimby et al. (1999). The Stabileze formulation technique was simple to perform and the product easy to store. In the dry form, the formulation consisted of an aggregate of small clumps, measuring a maximum of 5 mm in diameter. When the dry formulation was placed on PDA, *N. neomacrospora* mycelium emerged from these clumps within 48 hours. The dry formulation could be suspended fairly easily in water at 1% solution and did not clog the hand held atomizer. The larger clumps took longer to suspend in water; these could have been removed if the sieve method described by Quimby et al. (1999) had been used.

The PDA plates sprayed with the Stabileze solution used in the field trial confirmed that the conidia were viable. Within one week, numerous colonies of *N. neomacrospora* had established for the Stabileze used on treatments A, B, and C. The control Stabileze (no spores) used for treatments J, K and L did not give rise to *N. neomacrospora* colonies.

3.3.2 Symptoms and signs of *N. neomacrospora* in the field

Since *N. neomacrospora* infects the *A. tsugense* swelling, it is difficult to monitor early stages of disease development. For the first 2 months following treatment application, there were no evident symptoms or signs that infection of *A. tsugense* by *N. neomacrospora* had occurred.

Sporodochia-like structures were observed on many experimental units later on in the field trial. At no time throughout the trial were perithecia observed on any of the experimental units.

All *A. tsugense* infections showed a decline in the total number of healthy *A. tsugense* shoots throughout the trial period. The percentage of dead and diseased *A. tsugense* shoots remained below 25 % for all treatments until 6 months after inoculation. At this time, some treatments began to show an increase in broken, diseased and dead shoots. Diseased *A. tsugense* shoots did not show localized disease symptoms, but rather the entire *A. tsugense* shoot became “mummified” and turned dark brown as the tissues died. In some cases, the dead *A. tsugense* shoots remained attached to the swellings (Figure 3.1, 3.2). It is speculated that bark necrosis and girdling of the *A. tsugense* swelling contributed to reduced *A. tsugense* shoot vigour.



Figure 3.1. A treated *A. tsugense* swelling at 2 weeks (A) and 9 months (B) after treatment. Treatment involved wounding the *A. tsugense* swelling and applying inoculum (treatment C). Infection by *N. neomacrospora* was confirmed.



Figure 3.2. A treated *A. tsugense* swelling at 2 weeks (A) and 9 months (B) after treatment. Treatment involved applying inoculum to unwounded *A. tsugense* swellings (treatment A). Infection by *N. neomacrospora* was not confirmed.

3.3.3 Lost experimental units 10 months following inoculation

Across the large field trial period, 6 experimental units were lost (Table 3.4).

Table 3.4. The number of *A. tsugense* (A. t.) infections prior to treatment and remaining at termination of the trial.

Treatment	Number of A. t. infections prior to treatment	Number of lost A. t. infections	Number of A. t. infections at termination of the trial
A	35	2	33
B	35	0	35
C	35	2	33
J	35	0	35
K	35	2	33
L	35	0	35
N	27	0	27

Death of the *A. tsugense* swelling as a result of self-pruning of the host branch was rare. Selection of experimental units prior to treatment application discriminated against *A. tsugense* infections on lower hemlock branches that were prone to self-pruning. As a result, few (<1 %) of the *A. tsugense* swellings were lost to self-pruning. Instances where the host branch died *beyond* the *A. tsugense* swelling were much more common and were caused by girdling of the swelling.

Since *N. neomacrospora* attacks the *A. tsugense* swelling, and because its mechanism to reduce *A. tsugense* vigour is poorly understood, *A. tsugense* infections killed by girdling or host branch death were not removed from data analysis. Dead or girdled *A. tsugense* swellings were screened the same way as non-girdled *A. tsugense* branches. The occurrence of girdling was not used as a measure of successful infection by *N. neomacrospora*.

Squirrel damage was also minimal in the field trial (<1%) and therefore swellings affected by such damage also remained in data analysis. A windblown tree fell onto one

hemlock host containing several experimental units, and some of these units were lost. Tags from some other experimental units were also lost.

3.3.4 Importance of wounding for establishment of *N. neomacrospora*

During destructive sampling, there appeared to be a strong association between staining of the wood in the *A. tsugense* swelling and bark necrosis. Staining has also been observed in diseased *Nectria galligena* apple wood in apple orchards (McCracken et al., 2003). Bark necrosis and cankering of the *A. tsugense* swelling associated with *N. neomacrospora* infection may provide a habitat for stain and decay fungi (Baranyay, 1966) It is not known if *N. neomacrospora* or another fungal species is responsible for the staining observed in this trial.

In addition to staining, numerous insects were found within the bark of destructively sampled *A. tsugense* swellings. Many of the *A. tsugense* swellings had insect cavities, and 9 of the 132 swellings still had larval insects in the bark. These insects caused extensive bark necrosis within the *A. tsugense* swelling. Byler, Cobb and Parameter (1972b) also noted the occurrence of insects in *N. neomacrospora* colonized rust galls and suggested that insects may serve as vectors for some fungi. No attempt was made to identify the species of insects found on and within the *A. tsugense* swellings.

3.3.4.1 Extent of bark necrosis

The extent of bark necrosis for each destructively examined treatment is shown in Figure 3.3. Statistical analysis of treatments A, C, J, and L using a two-way analysis of variance on ranks found a significant interaction between wound treatments and inoculum treatments was also observed ($P=0.015$). Further analysis detected significant differences between treatments C and L and A and C ($P<0.001$, $P<0.001$, respectively). Statistical analysis of treatments A, B, J and K failed to detect significant differences between wound treatments or a significant interaction between wound and inoculum treatments. There was, however, a significant difference between inoculum treatments ($P=0.013$).

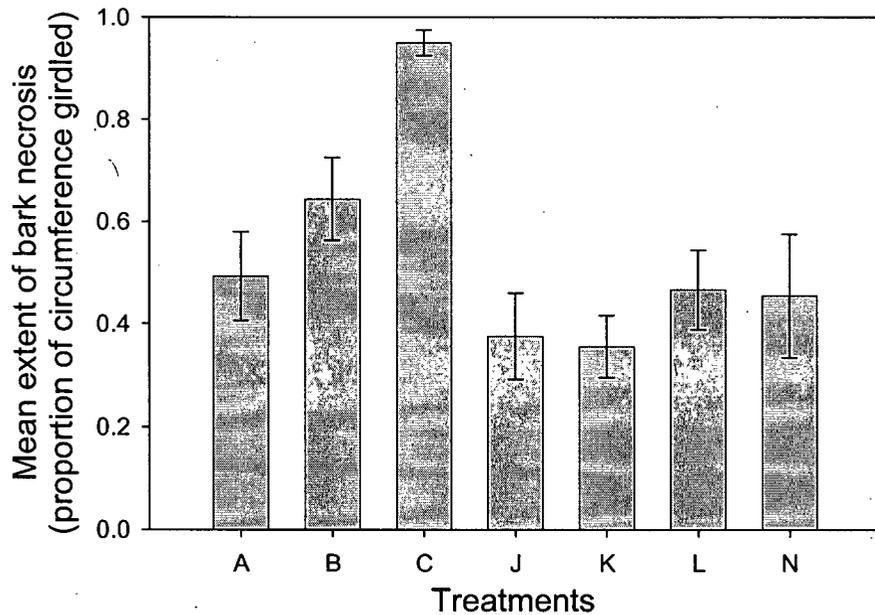


Figure 3.3. Mean extent of bark necrosis for *A. tsugense* swellings collected 10 months after treatment. Bars measure standard error of the mean.

Eighty-five percent of the *A. tsugense* swellings from treatment C were completely girdled or had girdled side branches (data not shown). This is much higher than the proportion of girdled *A. tsugense* swellings observed for treatments A, B, and the respective control (55%, 55%, and 45%, respectively).

3.3.4.2 Proportion of *A. tsugense* with sporodochia

The proportion of *A. tsugense* swellings bearing sporodochia (presence or absence) 10 months after treatment is shown in Figure 3.4. Lumped chi-square analysis of treatments A, B, C, J, K and L found significant differences between treatments ($P < 0.001$). Significantly more *A. tsugense* swellings from treatment C had sporodochia when compared to treatments A and L ($P = 0.014$, $P < 0.001$ respectively). Treatments A and B did not significantly increase the occurrence of sporodochia when compared to their respective controls (treatments J and K). The occurrence of sporodochia for treatment N were not significantly greater than that measured for treatment J. No sporodochia were observed for the control treatments J, K or L at 10 months after treatment.

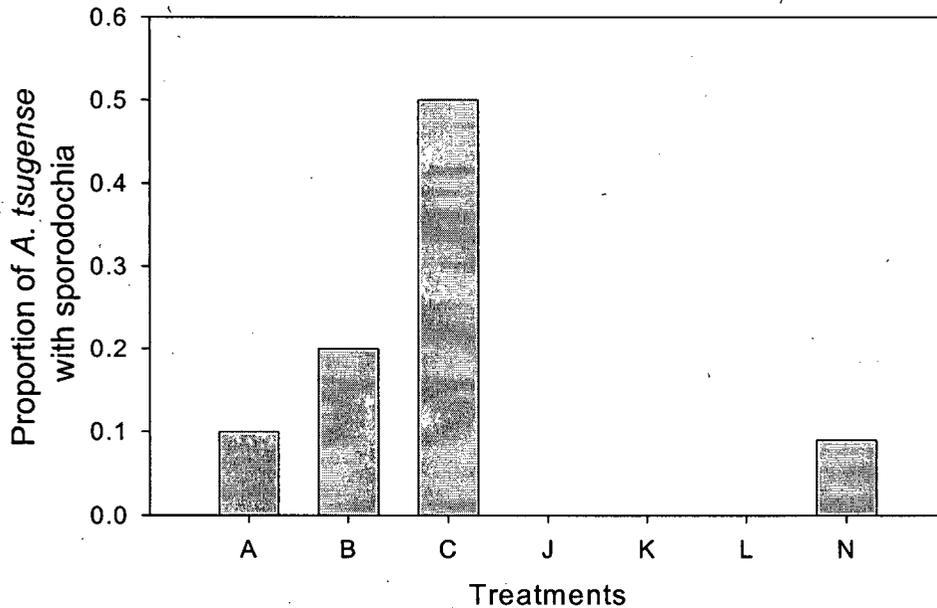


Figure 3.4. Proportion of *A. tsugense* swellings collected 10 months after treatment bearing confirmed sporodochia.

The occurrence of sporodochia observed during the large field trial period was looked at both as cumulative and non-cumulative data. Since the occurrence of sporodochia observed in the field was based on observational data and not confirmed, statistical analysis of the data was not done.

For the cumulative data, the occurrence of sporodochia was treated as additive from one assessment to the next, such that all units that bore sporodochia at any time were considered to have sporodochia. Treatments B and C had more sporodochia than their respective controls at 3.5, 6 and 9 months (Figure 3.5). Treatment A did not appear to increase the occurrence of sporodochia when compared to its respective control.

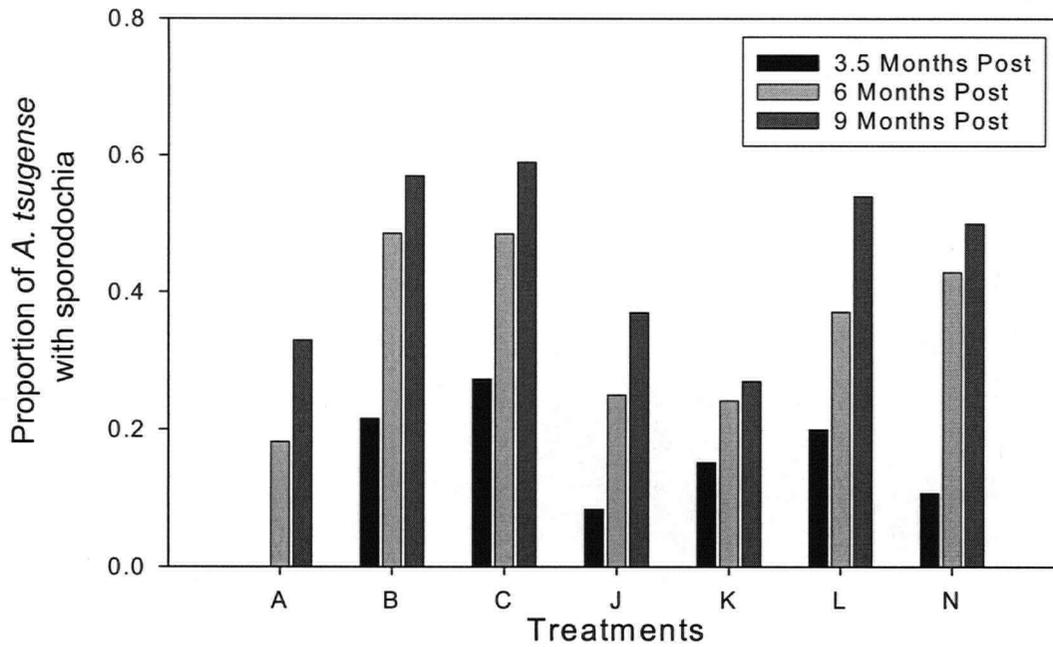


Figure 3.5. Cumulative proportion of *A. tsugense* swellings bearing sporodochia 3.5, 6 and 9 months after treatment.

The non-cumulative approach looked at the occurrence of sporodochia at each assessment and did not consider data from previous assessments. No apparent trends were observed between treatments for non-cumulative occurrence of sporodochia (data not shown). However, the non-cumulative occurrence of sporodochia for all treatments combined increased over time (Figure 3.6). The greatest increase in the proportion of *A. tsugense* swellings with sporodochia occurred 2-4 months after treatment (between October and December). The non-cumulative occurrence of sporodochia was highest in May at the end of the large field trial period.

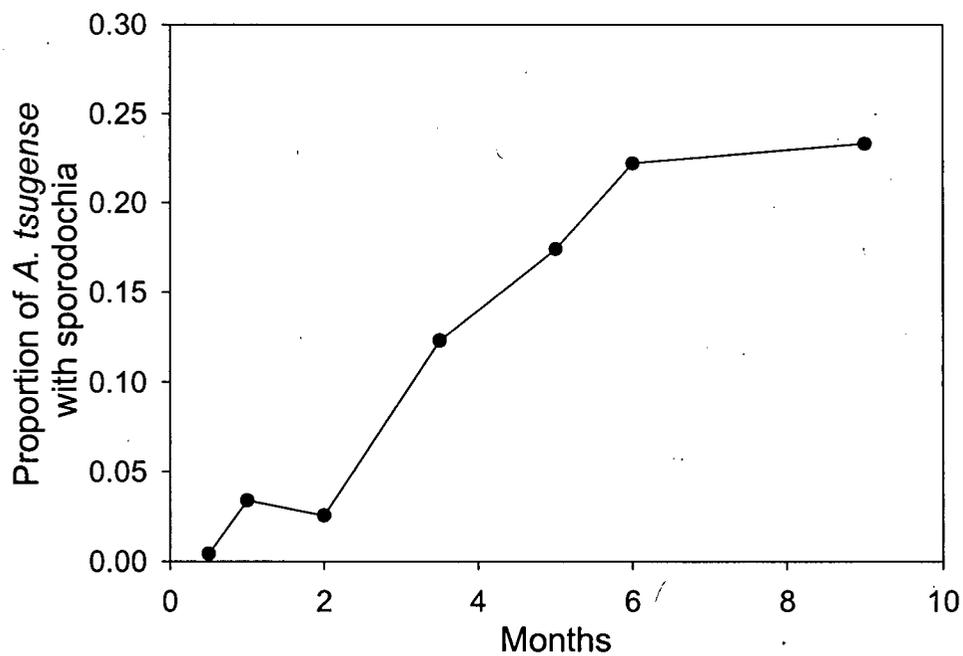


Figure 3.6. The non-cumulative proportion of *A. tsugense* swellings bearing sporodochia. All treatments are grouped together for this data.

3.3.4.3 Re-isolation of *N. neomacrospora* from *A. tsugense* swellings

Results for re-isolation of *N. neomacrospora* from *A. tsugense* swellings are shown in Figure 3.7. Chi-square analysis detected significant differences between treatments A, B, C, J, K and L ($P = 0.003$). Treatment A had much higher re-isolation of *N. neomacrospora* than expected based on the extent of bark necrosis and the occurrence of sporodochia, and was significantly greater than its respective control ($P = 0.048$). Treatments B and C also resulted in significantly higher re-isolation of *N. neomacrospora* than their respective controls ($P=0.022$, $P = 0.025$, respectively). Treatment C had the highest re-isolation of *N. neomacrospora* of all treatments. The difference between treatment J and treatment N was negligible.

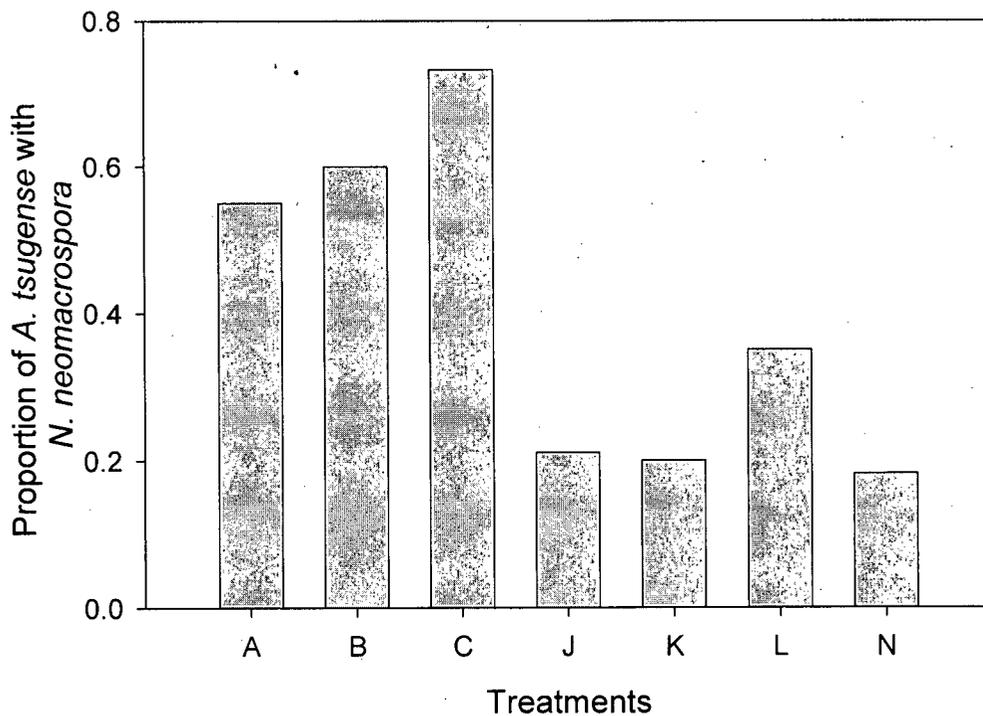


Figure 3.7. Proportion of *A. tsugense* swellings from which *N. neomacrospora* was successfully re-isolated 10 months after treatment.

Comparison of the confirmed occurrence of sporodochia at the end of the field trial and successful re-isolation of *N. neomacrospora* from the destructively sampled *A. tsugense* swellings suggested that re-isolation may fail to detect as much as 30% of infected *A. tsugense* swellings (Table 3.5). Sporodochia were absent from 78% of *A. tsugense* swellings from which *N. neomacrospora* was successfully re-isolated. Chi-square analysis detected significant differences between groups ($P=0.020$).

Table 3.5 Number of destructively sampled *A. tsugense* swellings at 10 months with and without sporodochia and successful re-isolation of *N. neomacrospora*.

	Sporodochia Observed	Sporodochia Not Observed
Re-Isolation Successful	12	43
Re-Isolation Unsuccessful	5	72

When the extent of bark necrosis was broken down into 4 classes of 25% each and plotted against the proportion of *A. tsugense* swellings with confirmed *N. neomacrospora* infection for each class, a strong linear relationship emerged (Figure 3.8). The best linear model fit was $Y = -0.034 - 0.189X$, where Y = the proportion of the *A. tsugense* swellings with confirmed infection and X = the class of bark necrosis ($r^2 = 0.93$). Confirmed *N. neomacrospora* infection was based on successful re-isolation of *N. neomacrospora* and/or confirmed sporodochia at the end of the field trial.

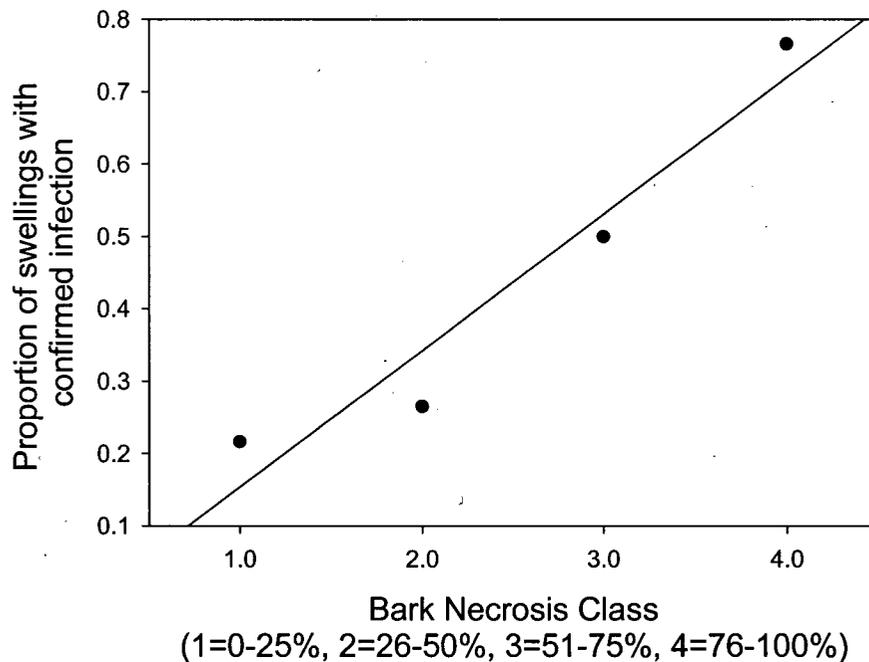


Figure 3.8. Linear relationship of the extent of bark necrosis and the proportion of *A. tsugense* swellings from which *N. neomacrospora* was successfully re-isolated. Regression analysis of $Y = Y_0 + aX$ found $r^2 = 0.93$.

3.3.5 Impact of *N. neomacrospora* on *A. tsugense* vigour

3.3.5.1 Number of healthy *A. tsugense* shoots for confirmed and unconfirmed groups

Arceuthobium tsugense swellings were regrouped into confirmed and unconfirmed groups regardless of treatment. The confirmed group (N=60) consisted of the destructively sampled *A. tsugense* swellings from which *N. neomacrospora* was successfully re-isolated or which bore sporodochia at 10 months. The unconfirmed group (N=72) consisted of the remainder of destructively sampled *A. tsugense* swellings which failed to successfully re-isolate *N. neomacrospora* and did not bear sporodochia. In the case of shoot analysis, cut shoot treatments (B and K) were removed from analysis because the treatment process itself may have influenced the variable of interest (i.e. number of shoots). Analysis of the number of healthy *A. tsugense* shoots 9 months after treatment found significant differences between confirmed and unconfirmed groups (Figure 3.9); ($P=0.025$). The confirmed group had 1.63 fewer shoots than the unconfirmed group, showing a 38% decrease.

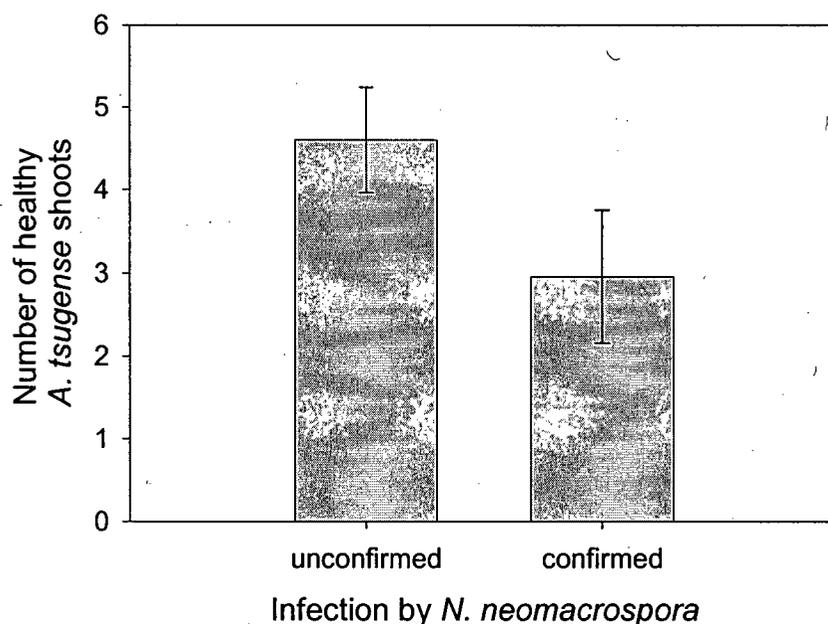


Figure 3.9. The mean number of healthy *A. tsugense* shoots 9 months after treatment for unconfirmed and confirmed groups. Cut shoot treatments were removed from analysis. Bars measure standard error of the mean.

A general decline in the total number of *A. tsugense* shoots was observed for both confirmed and unconfirmed groups throughout the trial period (Figure 3.10). The largest decrease in the number of healthy *A. tsugense* shoots occurred between pre-treatment assessment (0 months) and 0.5 months after treatment.

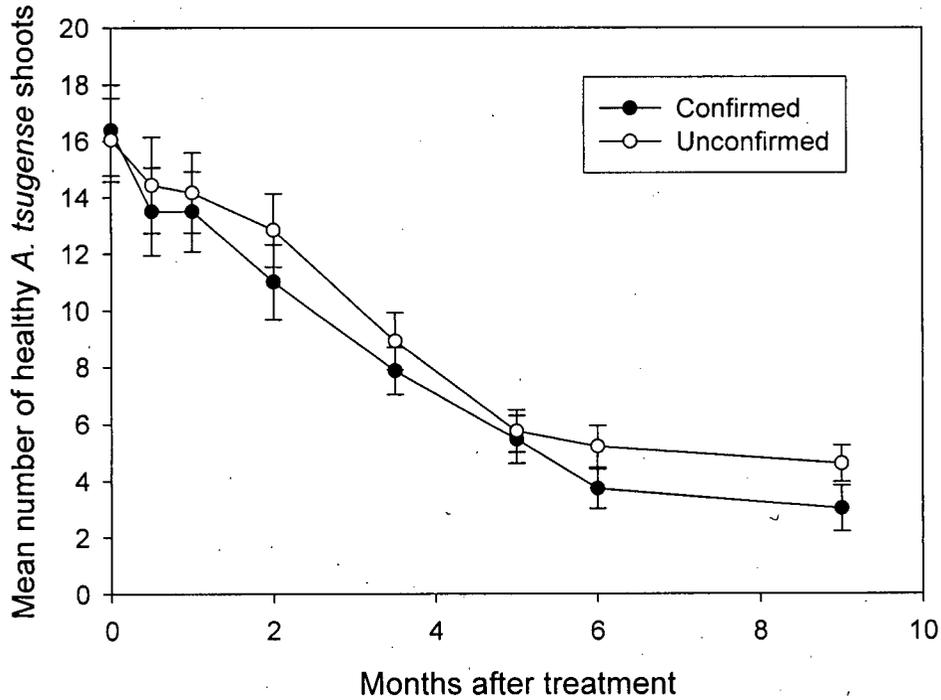


Figure 3.10. Mean number of healthy *A. tsugense* shoots per swelling for unconfirmed and confirmed *N. neomacrospora* infected groups. Bars measure standard error of the mean.

3.3.5.2 Proportion of *A. tsugense* infections with zero shoots for unconfirmed and confirmed groups

The confirmed group had a significantly greater proportion of *A. tsugense* infections with no shoots than the unconfirmed group 10 months after treatment (Figure 3.11); ($P=0.002$). Approximately 57% of the *A. tsugense* infections from the confirmed group did not have shoots, and 33% of the *A. tsugense* infections from the unconfirmed group did not have shoots.

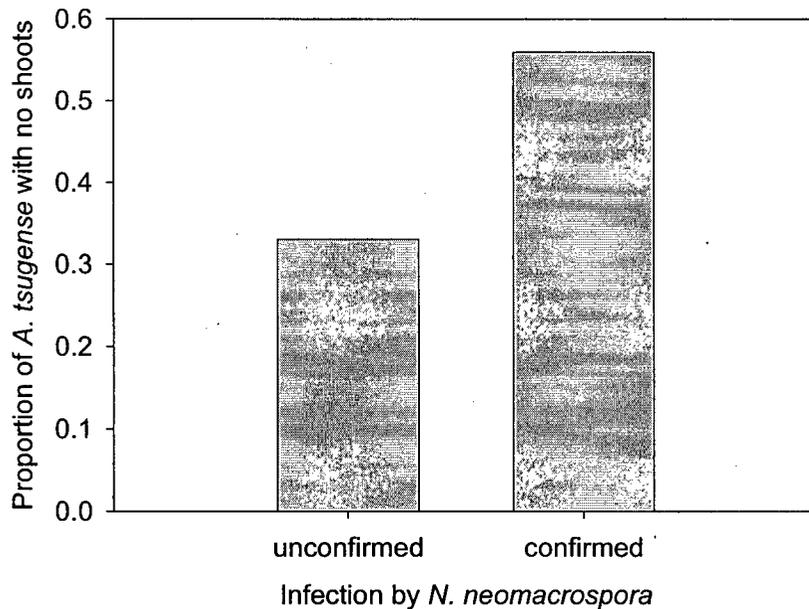


Figure 3.11. Proportion of *A. tsugense* infections with no shoots 10 months after treatment for unconfirmed and confirmed groups.

3.3.5.3 Extent of bark necrosis and girdling of *A. tsugense* swellings for unconfirmed and confirmed groups

At 10 months after treatment, the mean extent of bark necrosis for the confirmed group (73%) was nearly twice as great as that observed for the unconfirmed group (37%). Statistical analysis using a one-way analysis of variance on ranks detected significant differences between the two groups ($P<0.001$).

Chi-square analysis of the proportion of girdled *A. tsugense* infections detected a significant difference between the confirmed and unconfirmed groups ($P < 0.001$); (Figure 3.12). The confirmed group had just over two times as many girdled *A. tsugense* swellings (68%) when compared to the unconfirmed group (33%).

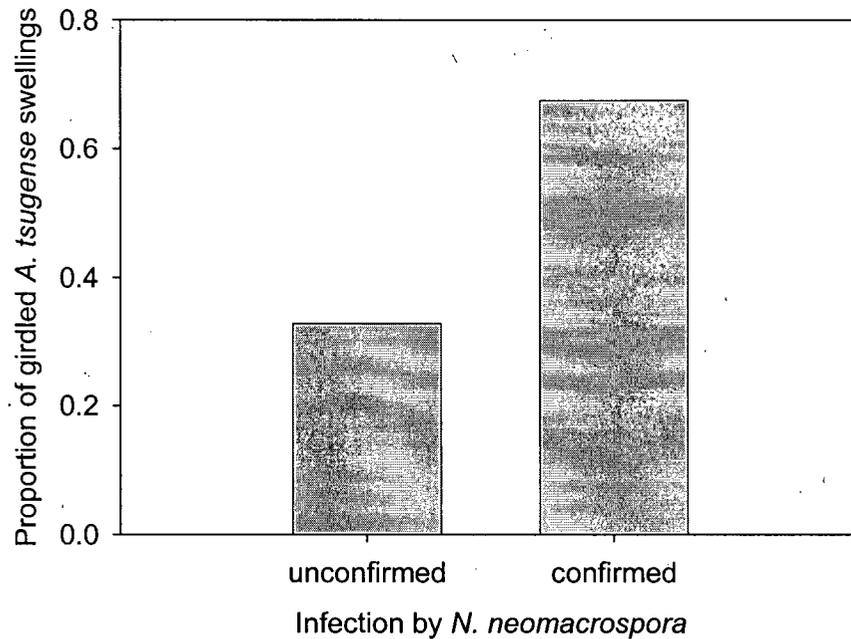


Figure 3.12. Proportion of girdled *A. tsugense* swellings 10 months after treatment for unconfirmed and confirmed groups.

3.4.0 Discussion

Wound-related response to fungal attack is a complex process that involves necrosis (death) of host tissue at the site of pathogen attack (Woodward and Pearce 1988; Biggs, Merrill, and Doris 1984). Successful pathogens are able to initiate infection and overcome host defence and compartmentalization. Although there is much potential for using necrotizing fungi as a biological control strategy, only a few have been evaluated.

Early work by Byler, Cobb and Parmeter (1972a; 1972b) found the necrotizing fungus *Neonectria neomacrospora* was able to infect and kill galls caused by *Peridermium harknessii* on Bishop pine and Monterey pine. Further investigation found that this fungus was a virulent pathogen of dwarf mistletoe infected pine tissues, but only weakly pathogenic to healthy conifer tissue (Byler and Cobb 1972).

Nectria ditissima Tul., a wound pathogen that attacks red alder (*Alnus rubra* Bong.), is another necrotizing fungus that has been investigated as a potential biological control agent (Dorworth 1995; Dorworth et al. 1996). Dorworth (1995) developed an inoculation strategy for this fungus (ALDERKILL™), and registered the formulated form of *N. ditissima* as PFC-MYCOCHARGE®. Inoculation trials showed that the inoculation strategy was able to produce 100% infection, and that once infection was established, cankers longer than 0.5 m developed in 30 months (Dorworth 1995). Although disease symptoms caused by *N. ditissima* infection on red alder are slow to develop, the PFC-MYCOCHARGE technique is still recommended for pre-commercial stand improvement.

Establishment of an inoculation procedure for *N. neomacrospora* is necessary before it can be further assessed as a biological control agent for hemlock dwarf mistletoe. While there has been some previous inoculation work with *N. neomacrospora* on dwarf mistletoe (Byler and Cobb 1972; Funk, Smith and Baranyay 1973; Smith and Funk 1980), this is the first large field trial to use different wounding techniques. Results from this trial were broken down according to two main objectives: to determine whether wounding is required for infection by *N. neomacrospora* to occur and to measure the impact of *N. neomacrospora* infection on *A. tsugense* shoot health and number and swelling health.

Because of concern for secondary infection of experimental units, the large field trial was terminated 9 months after treatment. Sporodochia were common at the field site and occurred on experimental units and other *A. tsugense* swellings that were not used in the trial. Perithecia were also observed on *A. tsugense* swellings that were not used in the trial. At 9 months, 50% or more of the *A. tsugense* swellings from treatments B, C, L and N had had sporodochia at one point in time (Figure 3.5). *Neonectria neomacrospora* was successfully re-isolated from 16-35% of the *A. tsugense* swellings from the uninoculated treatments (J, K and L), indicating that some background infection had occurred (Figure 3.7). Early termination of the field trial prevented the long-term study of infection by *N. neomacrospora* on *A. tsugense* vigour.

3.4.1 Stabileze formulation

Smith and Funk (1980) stressed the need for an improved inoculation technique to assess *N. neomacrospora* as a potential biological control agent for *A. tsugense*. Because of this, it was decided that the inoculum source for the field trial would consist of conidia from the anamorph stage of *N. neomacrospora* formulated in Stabileze. Formulation can improve spore viability once applied in the field and permits long-term storage of inoculum (Quimby et al. 1996; Boyette 1994).

Results from the large field trial show that Stabileze formulated conidia of *N. neomacrospora* permitted infection of the *A. tsugense* swelling. Application of formulated conidia onto *A. tsugense* swellings increased infection by 39-40 % based on re-isolation data (Figure 3.6). This level of infection is higher than the 25% increase in infection observed in the non-formulated inoculation trial conducted by Smith and Funk (1980). It is not clear, however, if differences are due to formulation of the spores or other experimental factors. A trial that directly compares the efficacy of Stabileze formulated spores versus non-formulated spores is required before any definitive conclusions about Stabileze can be made.

3.4.2 Importance of wounding for establishment of *N. neomacrospora*

The extent of bark necrosis, occurrence of sporodochia, and successful re-isolation of *N. neomacrospora* from destructively sampled *A. tsugense* swellings were

used to determine the requirement of wounding for *N. neomacrospora* infection. Because bark necrosis is much more useful in determining *N. neomacrospora* infection than the occurrence of sporodochia or re-isolation of *N. neomacrospora*, it was given precedence over the other two measurements.

Bark necrosis of the *A. tsugense* swelling is a symptom commonly associated with *N. neomacrospora* infection that often leads to cankering and girdling of the swelling (Funk, Smith and Baranyay 1973; Smith and Funk 1980). It is advantageous to use bark necrosis to quantify *N. neomacrospora* infection because it is easy to measure and is consistently correlated with *N. neomacrospora* infection. Although there was some bark necrosis in almost all *A. tsugense* swellings regardless of treatment, a strong, linear relationship between different classes of bark necrosis and the proportion of *A. tsugense* swellings from which *N. neomacrospora* was successfully re-isolated was observed ($r^2=0.93$); (Figure 3.8).

The occurrence of sporodochia and re-isolation of *N. neomacrospora* from the *A. tsugense* swelling are useful in that they confirm *N. neomacrospora* infection, but failure to detect sporodochia or to re-isolate *N. neomacrospora* does not necessarily mean infection has not occurred. Comparison of those *A. tsugense* swellings from which sporodochia were observed to those from which *N. neomacrospora* was successfully re-isolated verified that neither of these measurements were perfect indicators of infection (Table 3.5). Sporodochia were observed on only 21% of the *A. tsugense* swellings from which *N. neomacrospora* was re-isolated, and *N. neomacrospora* was re-isolated from only 70% of the *A. tsugense* swellings that had sporodochia.

In future trials, it may be worthwhile to develop molecular markers for detection of *N. neomacrospora* in the *A. tsugense* swelling. Recent work by Langrell (2002) developed a species-specific PCR technique for detection of *Neonectria galligena* from wood of young apple trees. A similar technique could be modified for detection of *N. neomacrospora* in the bark and wood of *A. tsugense* swellings.

For the wounded, inoculated treatment (C), the extent of bark necrosis was greater than the sum of wounding and inoculation alone (Figure 3.3). Statistical analysis detected a significant interaction between wounding and inoculation ($P=0.015$), suggesting that this treatment caused the greatest amount of infection. The extent of bark necrosis for the

unwounded, inoculated treatment (A) and the cut shoots, inoculated treatment (B) was only marginally higher than the controls.

The non-cumulative occurrence of sporodochia at 10 months and re-isolation of *N. neomacrospora* concur with these findings (Figures 3.4, 3.7). The wounded, inoculated treatment (C) had significantly more sporodochia than the other treatments and had the highest success of re-isolation of *N. neomacrospora*. The occurrence of sporodochia and successful re-isolation of *N. neomacrospora* for the cut shoots, inoculated treatment (B) were slightly higher but not significantly different from the unwounded, inoculated treatment (A).

Based on these findings, the wounded, inoculated treatment (C) had the greatest impact on *N. neomacrospora* infection. Although there was some indication that infection was occurring in the other inoculated treatments, the impact of these treatments on *A. tsugense* swelling bark necrosis was marginal in comparison to the wounded, inoculated treatment (C). There was no strong evidence that *N. neomacrospora* is able to colonize the *A. tsugense* swelling via cut *A. tsugense* shoots.

These findings do not concur with an inoculation study done by Funk, Smith and Baranyay (1973), where *N. neomacrospora* successfully established after applying mycelial inoculum to undisturbed *A. tsugense* basal cups or young shoots. However, inoculated *A. tsugense* swellings in the trial conducted by Funk, Smith and Baranyay (1973) were monitored for almost 2 years after treatment and some *A. tsugense* swellings did not show signs of infection until as late as 21 months.

The disease development of *N. neomacrospora* is slow and can take a year or longer to establish symptoms (Funk and Baranyay 1973). The long-term impact of *N. neomacrospora* infection (i.e. 1 year or longer) was not measured in the large field trial because it was terminated early at 9 months. Although the extent of bark necrosis for the unwounded, inoculated treatment (A) was not significant, the treatment had 39% more *N. neomacrospora* re-isolation success than its respective control ($P=0.048$); (Figure 3.7). It is possible that development of symptoms and signs of disease (i.e. bark necrosis, sporodochia) in the unwounded, inoculated treatment (A) is slower than the wounded, inoculated treatment (C). The impact of the unwounded, inoculated treatment (A) on *A.*

tsugense shoot health may therefore be larger in the long term when disease has had sufficient time to develop.

The large field trial was terminated early because of the high occurrence of background *N. neomacrospora* inoculum. Ideally, the large field trial should have been run on a site free of natural *N. neomacrospora* background inoculum. Despite efforts, a site with high *A. tsugense* infection and little to no *N. neomacrospora* infection could not be found. Several sites on southern Vancouver Island were assessed, and there appeared to be a strong correlation between high levels of *A. tsugense* infection and the presence of *N. neomacrospora*. It was necessary to choose a site with high *A. tsugense* infection in order to have a sufficient number of experimental unit replicates.

3.4.3 Effect of *N. neomacrospora* infection on *A. tsugense* vigour

For the second objective, experimental units were re-grouped into two groups (confirmed and unconfirmed) regardless of treatment. The confirmed group consisted of *A. tsugense* swellings from which *N. neomacrospora* had been successfully re-isolated, or which had sporodochia at the end of the trial. The unconfirmed group consisted of all other *A. tsugense* swellings and may have contained *N. neomacrospora* infected *A. tsugense* swellings that escaped detection.

The impact of *N. neomacrospora* infection on *A. tsugense* vigour was determined by measuring the number of healthy *A. tsugense* shoots, the proportion of *A. tsugense* swellings with zero shoots, the extent of bark necrosis, and the proportion of girdled *A. tsugense* swellings for confirmed and unconfirmed groups.

Measuring the number of healthy shoots, in particular, was important because it estimated how much *N. neomacrospora* infection reduced *A. tsugense* reproduction. Since *N. neomacrospora* acts perennially, shoot reduction is expected to continue and negatively impact the ability of *A. tsugense* to produce inoculum the year(s) following treatment. The impact of *N. neomacrospora* infection on the number of *A. tsugense* berries on female plants could not be measured directly because there was an insufficient number of healthy, female *A. tsugense* plants at the field site. In addition, since berries are produced annually, at least 1 complete year of data would be required before the impact of *N. neomacrospora* infection on the number of healthy *A. tsugense* berries could

be determined. *Arceuthobium tsugense* shoots are produced throughout most of the year and remain attached to the *A. tsugense* infection. It is not known if *A. tsugense* plants are pollen limited. The impact of *N. neomacrospora* infection on the extent of bark necrosis and the occurrence of girdling were measured because these factors are expected to impact the number and health of *A. tsugense* shoots.

Although all *A. tsugense* swellings lost shoots over the trial period (Figure 2.19), the confirmed group lost significantly more *A. tsugense* shoots than the unconfirmed group ($P=0.025$); (Figure 3.10). In addition, more *A. tsugense* swellings from the confirmed group did not have *A. tsugense* shoots (Figure 2.20). Overall, infection of *A. tsugense* by *N. neomacrospora* reduced the number of healthy shoots by 1.6 shoots, or 38%. These data were similar to those observed by Funk, Smith and Baranyay (1973) who attributed a 30% reduction in the number of *A. tsugense* shoots to *N. neomacrospora* infection.

The confirmed group also had significantly more bark necrosis and girdling than the unconfirmed group (Figure 2.21). Although several *A. tsugense* swellings were completely girdled at the end of the trial, bark necrosis in other *A. tsugense* swellings was localized to one region of the swelling and had minimal impact on *A. tsugense* shoots in healthy parts of the swelling. Although parts of the *A. tsugense* swelling were girdled or had a large extent of bark necrosis, the *A. tsugense* swelling could still bear numerous healthy *A. tsugense* shoots in unaffected areas. As bark necrosis develops in infected *A. tsugense* swellings, the impact of *N. neomacrospora* on the number of healthy *A. tsugense* shoots is expected to increase.

3.4.4 Measuring the impact of treatment A on *A. tsugense* shoot vigour

At present, the unwounded, inoculated treatment (A) is the only feasible treatment that could be used in the field to control *A. tsugense*. The necessity of wounding for the other two treatments (B and C) makes them impractical for large-scale control. Wounding vectors, frost damage, and emerging *A. tsugense* buds and basal cups may provide some natural wound entry points for *N. neomacrospora* infection (Byler, Cobb and Parmeter 1972b). Insect galleries and larvae were observed in some of the

destructively sampled *A. tsugense* swellings, but the identity of the insects was not determined. Further research into manipulation of wound vectors to increase *N. neomacrospora* infection is required.

The impact of applying formulated conidia to unwounded *A. tsugense* swellings on the number of healthy *A. tsugense* shoots can be estimated via two routes. The first was simply to look at the average number of healthy *A. tsugense* shoots for the unwounded, inoculated treatment (A; 3.5 shoots) and compare that with its respective control (J; 5.9 shoots). In this case, applying inoculum to unwounded *A. tsugense* swellings reduced the number of *A. tsugense* shoots by an average of 2.4 shoots, or 41%. This is significantly different ($P=0.008$). However, the number of *A. tsugense* shoots observed for the respective control (J) was unusually high when compared to other uninoculated treatments (K, L and N). If the unwounded, inoculated treatment (A) were compared to the untreated treatment (N), the difference in number of shoots would not be significant (in this case, a 6% increase).

The second way to calculate the number of healthy shoots required two steps. The first step determined the proportion of *A. tsugense* swellings from the unwounded, inoculated treatment (A) and its respective control (J) that were infected by *N. neomacrospora*. In this case, *N. neomacrospora* was confirmed for 55% of the unwounded, inoculated treatment and 29% of the respective control (Table 3.3). Next, the impact of infection by *N. neomacrospora* on the number of healthy *A. tsugense* shoots was determined. All treatments were combined and re-grouped; the confirmed group had an average of 2.9 *A. tsugense* shoots and the unconfirmed group had an average of 4.6 *A. tsugense* shoots. By calculating the difference between the number of *A. tsugense* shoots expected for the unwounded, inoculated treatment (A) and the number of *A. tsugense* shoots expected for its respective control (J), the impact of the unwounded, inoculated treatment (A) on *A. tsugense* shoot vigour was estimated. Although this method can be considered "the long way around", it is more conservative in that infection by *N. neomacrospora* was confirmed. Also, this method does not depend on the unusually high number of shoots observed for the unwounded, uninoculated treatment (J).

Accordingly, using the first method, the unwounded, inoculated treatment (A) reduced the number of healthy *A. tsugense* shoots in the field by about 41%. This

measurement is much larger than that calculated by the second method, which suggested that the unwounded, inoculated treatment (A) reduced the number of healthy *A. tsugense* shoots about 14%. The true impact of the unwounded, inoculated treatment (A) on the number of *A. tsugense* shoots at 9 months is most likely in between these two values. The long-term impact of *N. neomacrospora* infection on *A. tsugense* shoot and swelling vigour is expected to be larger than that observed in the large field trial.

3.5.0 Closing Remarks

Neonectria neomacrospora is a unique biological control agent for *A. tsugense* because of its host specificity and its infection mechanism. This fungus attacks the swelling of *A. tsugense*, and has the potential of exerting a long-term negative impact on *A. tsugense* seed production. In some cases, *N. neomacrospora* may girdle and kill the *A. tsugense* swelling (Funk, Smith and Baranyay 1973).

To study the feasibility of using *N. neomacrospora* as a biological control agent of *A. tsugense*, a large field trial that involved inoculating *A. tsugense* swellings with Stabileze formulated conidia was conducted. Three treatments were assessed: inoculation of unwounded *A. tsugense* swellings, cut *A. tsugense* shoots, and wounded *A. tsugense* swellings. The data from this study suggest that wounding the *A. tsugense* swelling facilitates *N. neomacrospora* infection, resulting in the greatest amount of bark necrosis, sporodochia production, and successful re-isolation of *N. neomacrospora*. Although the unwounded, inoculated and cut shoots, inoculated treatments had greater re-isolation of *N. neomacrospora* than their respective controls, the impact of these treatments on *N. neomacrospora* infection was marginal when compared to the wounded, inoculated treatment. The unwounded, inoculated and cut shoots, inoculated treatments did not significantly increase bark necrosis or the occurrence of sporodochia when compared to their respective controls.

Unless a wounding mechanism is discovered, applying *N. neomacrospora* inoculum to unwounded *A. tsugense* swellings is the only economically feasible approach to control *A. tsugense*. Although the initial impact of the unwounded, inoculated treatment on *N. neomacrospora* infection appeared minor, it cannot be dismissed as a

potential biological control method. It is possible that disease development (i.e bark necrosis, sporodochia) is slower for the unwounded, inoculated treatment, and that the long-term impact on *A. tsugense* vigour will be greater than that observed in the large field trial.

Results from the trial also suggest that, once established, *N. neomacrospora* can significantly reduce *A. tsugense* shoot number by 38% at 9 months. Based on the extent of bark necrosis and girdling of the *A. tsugense* swellings observed during destructive sampling, the impact of *N. neomacrospora* infection on *A. tsugense* shoot vigour was lower than anticipated. Again, the extent of bark necrosis and girdling of the *A. tsugense* swelling are expected to have a greater, long-term impact on *A. tsugense* shoot production and the *A. tsugense* life cycle.

3.6.0 Future Research

In the future, it would be interesting to measure the long-term impact of *N. neomacrospora* infection on *A. tsugense* as well as other dwarf mistletoe species. The rate of bark necrosis spread in the *A. tsugense* swelling is of particular interest, as it will determine if *A. tsugense* is able to outgrow *N. neomacrospora* infection. It would also be worthwhile to see if *N. neomacrospora* isolates from different locations vary in levels of virulence and to confirm whether or not this fungus is able to infect other hosts such as *Abies*. Combining *N. neomacrospora* with another *A. tsugense* pathogen, such as *Colletotrichum gloeosporioides*, is also worth investigating. The study of natural *A. tsugense* wound vectors, which may allow for increased infection of *N. neomacrospora* in the field, is also of interest. In addition, the utilization of molecular markers for early detection of *N. neomacrospora* infection is an important contribution to study the host-pathogen interaction.

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Appendix I – Temperature on inoculation day (July 31st and August 6th, 2002) and immediately following for the small field trial

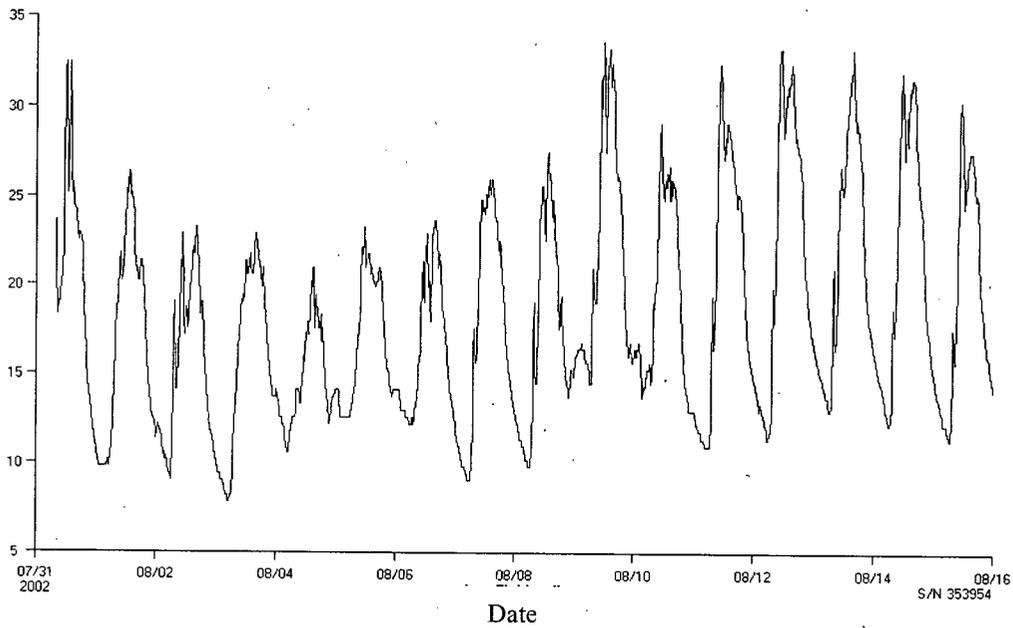


Figure appendix 1-1. Temperature on inoculation day and immediately following. Victoria, BC. Maximum temperature on inoculation day was 24°C on July 31st and 22.9°C on August 6th, 2002.

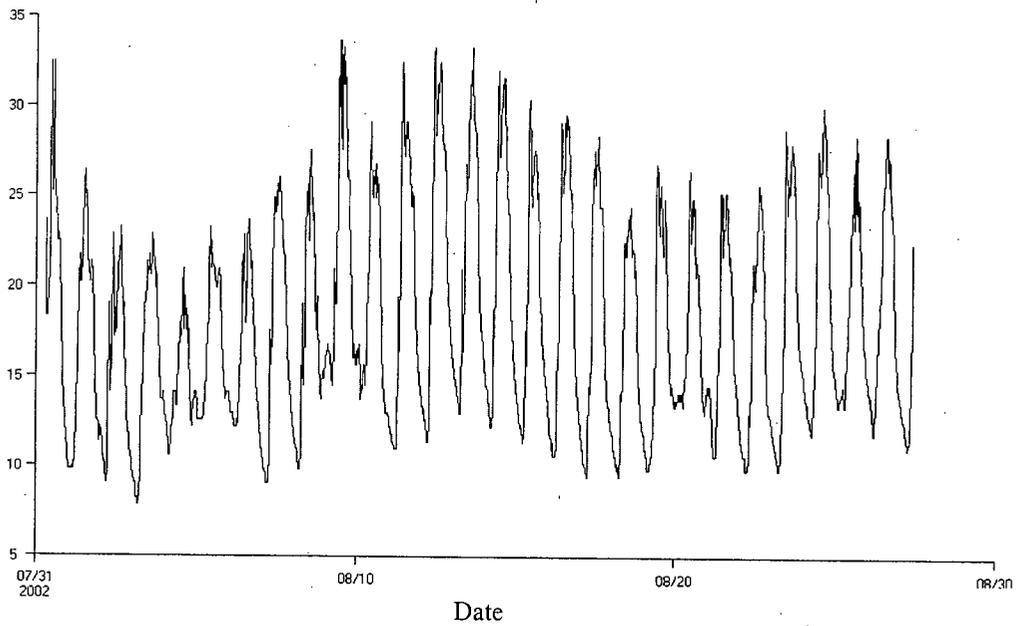


Figure appendix 1-2. Temperature at inoculation site from July 31st to August 30th. Victoria, BC.

Appendix II- Relative humidity (RH) on inoculation day (July 31st and August 6th, 2002) and immediately following for the small field trial

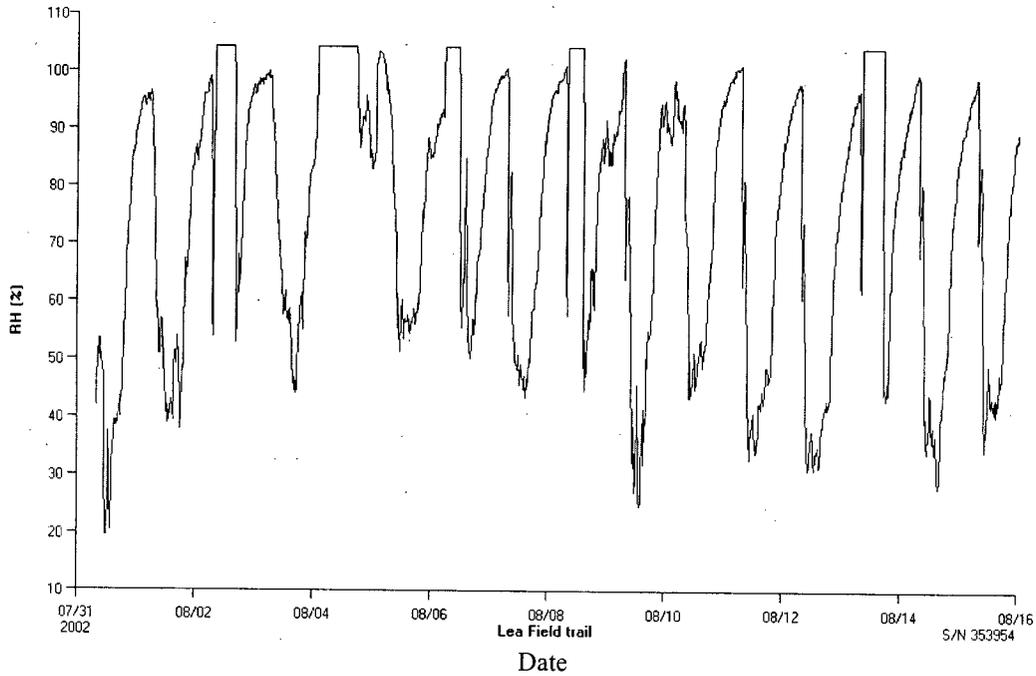


Figure appendix 4-1. Relative humidity on inoculation day and following. Victoria, British Columbia. Minimum relative humidity was 38.4% on July 31st and 50.2% on August 6th, 2002.

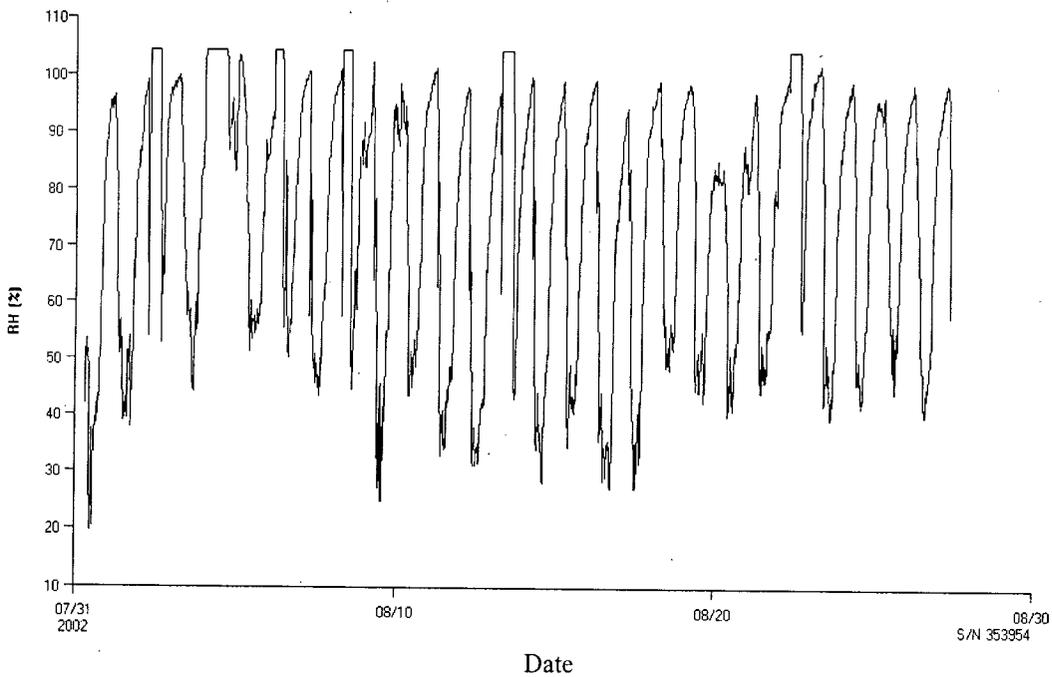


Figure appendix 4-2. Relative humidity at inoculum site from August 31st to October 27th, 2002. Near Parksville, British Columbia.

Appendix III- Temperature on inoculation day (August 31st) and immediately following for the large field trial

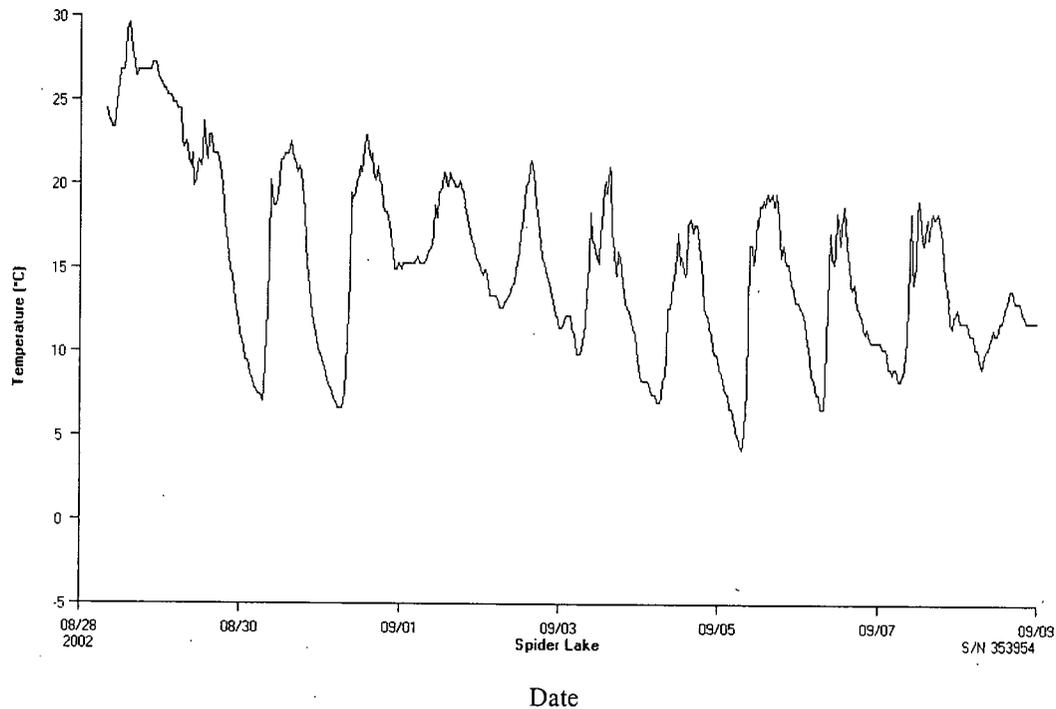


Figure appendix 3-1. Temperature on inoculation day and following. Near Parksville, British Columbia. Maximum temperature on inoculation day was 23.6°C at 1:35 PM.

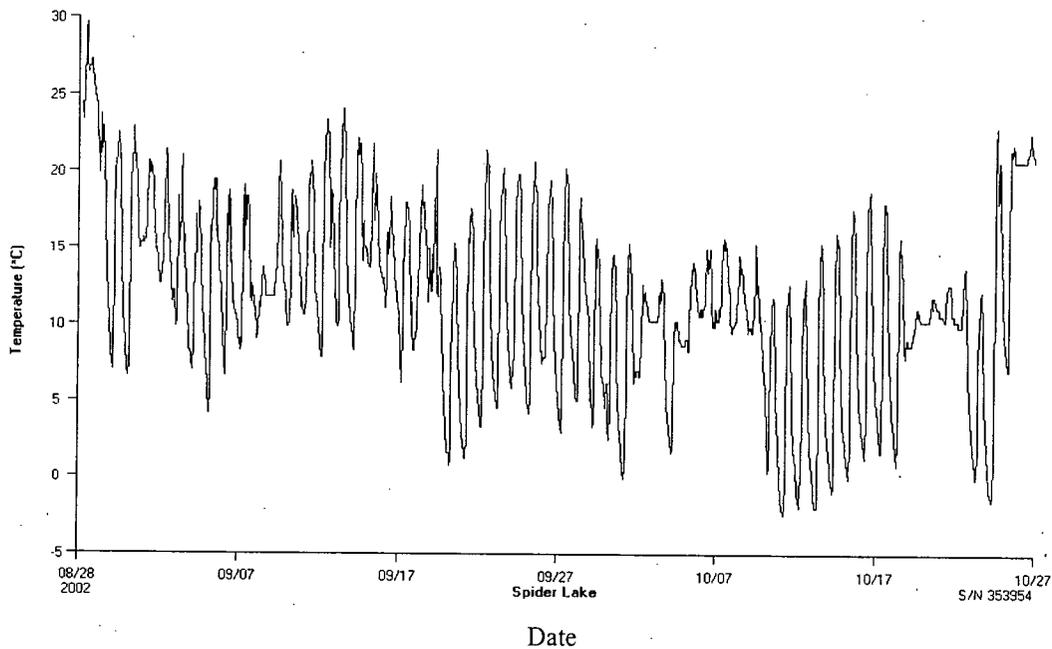


Figure appendix 3-2. Temperature at inoculation site from August 31st to October 27th, 2002. Near Parksville, British Columbia.

Appendix IV- Relative humidity (RH) on inoculation day (August 31st) and immediately following for the large field trial

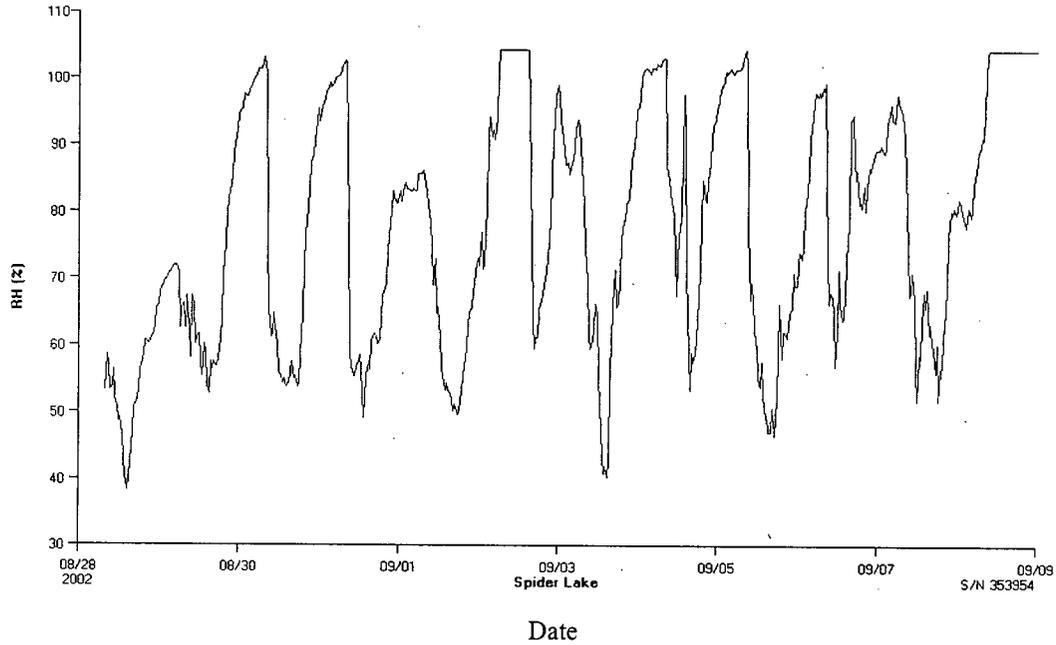


Figure appendix 4-1. Relative humidity on inoculation day and following. Near Parksville, British Columbia. Minimum relative humidity on inoculation day was 52.8% at 3:35 PM.

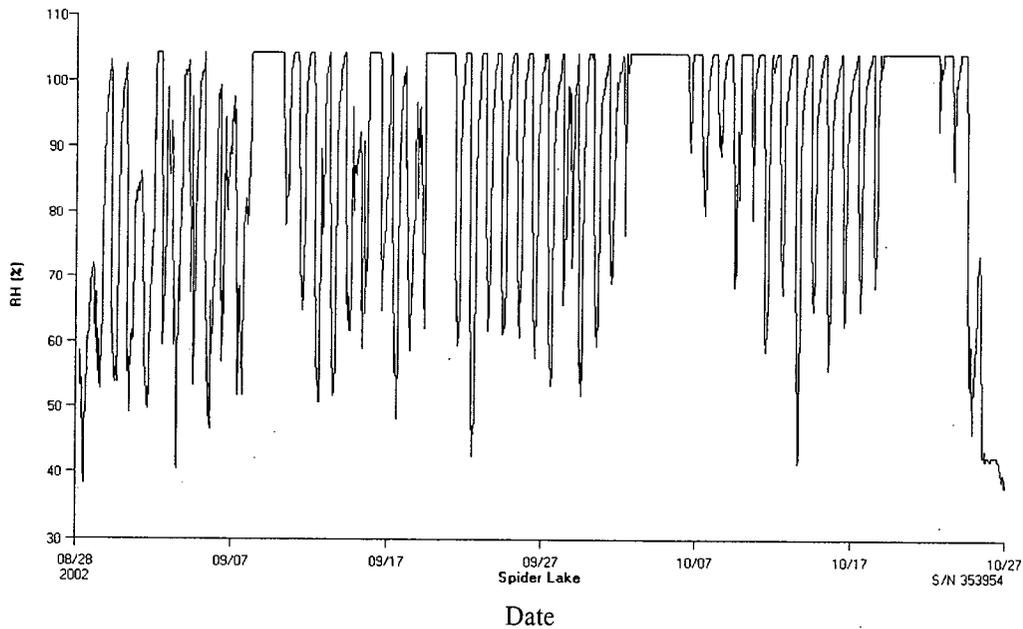


Figure appendix 4-2. Relative humidity at inoculum site from August 31st to October 27th, 2002. Near Parksville, British Columbia.