ASSESSMENT OF *COLLETOTRICHUM GLOEOSPORIOIDES* AS A BIOLOGICAL CONTROL OF HEMLOCK DWARF MISTLETOE (*ARCEUTHOBIUM TSUGENSE*)

by

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ABSTRACT

Use of an inundative biological control agent, *Colletotrichum gloeosporioides*, may provide an alternate method to control *Arceuthobium tsugense* (hemlock dwarf mistletoe) in the coastal forests of British Columbia, especially in variable retention areas, and in riparian and sensitive ecosystems. Variable retention practices can lead to in an increase in hemlock dwarf mistletoe infection, resulting in a loss of tree growth and vigor, reduction in wood quality, and tree death. Five Pacific Forestry Centre (PFC) *C. gloeosporioides* isolates were screened for conidia production, virulence, and lineal growth rate in culture. Results of this screening and a small field trial revealed that isolate PFC 2415 showed the most promising biological control agent characteristics. The highest number of *C. gloeosporioides* conidia was produced on the millet substrate compared to rice, slow oats and wheat bran.

A field trial using PFC 2415 in three treatments applied in late August 2002 (a Stabileze preparation sprayed on (1) intact mistletoe swellings and (2) on swellings on which all mistletoe shoots had been cut at 0.5 cm, and (3) a sucrose and gelatin preparation sprayed on intact mistletoe swellings) and their respective controls formulated without living inoculum was conducted near Nanaimo, BC. Stabileze® and sucrose-gelatin treatments formulated with *C. gloeosporioides* reduced the current berry crop by 16 to 36 percent respectively ($p<0.05$). While the results for shoots appeared promising, heavy background infection and/or secondary infection, especially on controls, limited the ability to detect clear treatment effects. Careful culturing from various live and dead host tissues showed that *C. gloeosporioides* was unable to invade and kill the mistletoe endophytic system within the living xylem and phloem of the host.

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1   CHAPTER 1-LITERATURE REVIEW

1.1   Introduction-Literature Review

Disease can be defined as any deviation in the normal functioning of a plant caused by some persistent agent (Manion 1991; Edmonds et al. 2000). Diseases include disorders that reduce growth, lower wood quality, or cause predisposition to attack by other agents, which may kill the tree. Both living organisms, called pathogens, and abiotic agents can cause disease (Manion 1991). Three critical factors or conditions must exist for a disease to occur: a susceptible host; a virulent pathogen; and the right mix of environmental conditions (Manion 1991; Agrios 1988; Edmonds et al. 2000).

Over the past decades researchers have also considered the spatial and temporal patterns in landscapes that influence the spread and pathogenicity of a pathogen (Castello et al. 1995). Host and pathogen spatial and temporal patterns are also closely connected to distribution, site relationships and incidence and severity, gene flow of the host and pathogen across a region which can significantly affect tree mortality (e.g. *Armillaria* and *Phytophthora*) (Castello et al. 1995). Landscapes also determine the biodiversity of host and species on site, the vigor of the tree through microclimate and mineral and water availability, the flow of genes as result of spatial location of trees and reservoirs of resistant species (Castello et al. 1995; Holdenrieder et al. 2004). Fragmentation of landscapes reduces migration and the ability of tree species to keep pace with climate change which can result in an increase of susceptibility to pathogens (Castello et al. 1995; Holdenrieder et al. 2004). Site factors such as slope, aspect, climatic factors, elevation, soil depth, drainage, solar radiation index can increase the predisposition of the host to disease (Castello et al. 1995; Holdenrieder et al. 2004). Ecological, evolution and pathogenicity processes of host pathogens are strongly linked to landscapes and may give foresters an insight to land use and forest sustainability (Castello et al. 1995; Holdenrieder et al. 2004).

Forest pathogens have both negative and positive effects on forest stands (van der Kamp 1991; Edmonds et al. 2000). As destructive agents, forest diseases can cause mortality and losses in growth and/or quality, and generally reduce the fitness of individual trees. In most cases, the effects of forest diseases are chronic rather than acute (Edmonds et al. 2000). On the positive side, forest diseases play an important role in the

cycling of carbon and mineral nutrients, and often increase landscape diversity (both spatial and temporal) and create special habitats (van der Kamp 1991; Edmonds et al. 2000; Gilbert 2002). The pathogen contributes to determining the rate of succession and the structure in the forest (Edmonds et al. 2000; Muir 2002). Environmental conditions also influence the rate of succession and stand structure, and composition of the tree species, soil, and tree spacing (Edmonds et al. 2000). In forests where major diseases affect early succession, species and overall succession may be accelerated (van der Kamp 1991; Edmonds et al. 2000; Muir et al. 2002).

Diseases also affect genetic variation in host susceptibility resulting in natural selection for greater resistance (Castello et al. 1995; Gilbert 2002). A successful host tree will adapt to a set of environmental conditions for a particular location or site (Holdenrieder et al. 2004). Disease types (common and/or rare) found on each site may vary from site to site. Tree provenances that co-exist on same site as a disease will adapt and become resistant to these site specific diseases (van der Kamp 1991; Gilbert 2002). The density of the host on a site can also influence the disease incidence and severity through direct effects on the host pathogen encounters or indirectly through by changing the environmental factors (Gilbert 2002). Disease incidence and severity can be influenced by 1) varying distances between host and the pathogen 2) frequency of available hosts for infection found through space and time 3) effect competition for host vigor and (4) other environmental stress factors found on a host tree site (Gibert 2002). Tree pathogens consequently induce genetic diversity in tree populations resulting in tree age diversity and also influencing the plant species found at a specific location with some diseases (Castello et al. 1995; Burdon 2001).

Forest health is generally considered to include a balance between tree growth, mortality, and regeneration; appropriate biological diversity; and the ability to withstand and recover from the impacts of various stressors, such as insect or disease outbreaks, adverse weather, or climate and air pollution (Edmonds et al. 2000). The lack of diversity and genotype variability of tree species can increase the risk of the health of a forest by increasing the susceptibility to disease (Gilbert 2002). Disturbances can create imbalances with specific organisms and trigger a series of complex interactions such as tree dieback, reduced growth and mortality (Gilbert 2002; McDonald 2003). A healthy

forest maintains and sustains desirable ecosystems and processes (Edmonds et al. 2000). It also includes the capability to meet management objectives over time (Edmonds et al. 2000; Muir 2002). Two factors cause major changes in forest health: disease and insects. Forest diseases in Canada are responsible for a timber loss equal to one-third of the annual harvest (Hall and Moody 1994).

In British Columbia, losses brought about by dwarf mistletoe are ranked in the top three diseases along with root rots and rusts (Nevill and Winston 1994; Wood and Sickle 1994). The commercial tree species that are most commonly infected in B.C by dwarf mistletoe species include *Tsuga heterophylla* (Raf.) Sarg., infected by western hemlock dwarf mistletoe *Arceuthobium tsugense* (Rosendahl) G.N. Jones; *Pinus contorta* Doug. ex Loud, infected by lodgepole pine dwarf mistletoe; *Arceuthobium americanum* Nutt. ex Engelm.; *Pseudotsuga menziesii* (Mirb.) Franco, infected by Douglas-fir mistletoe *Arceuthobium. douglasii* Engelm.; and *Larix occidentalis* Nutt., that is infected by western larch dwarf mistletoe, *Arceuthobium laricis* (Piper) St. John (Unger 1992; Muir et al. 2002).

1.2 *Arceuthobium* Species (Viscaceae)

1.2.1 Taxonomy and Distribution of *Arceuthobium*

Mistletoes are diverse groups of parasitic plants in the order Santales. Aerial shoots bear fruits possessing a viscid layer (Kuijt 1955, 1960; Hawksworth and Wiens 1996; Watson 2001). The two families of mistletoes are the Viscaceae and Loranthaceae. The mistletoes in the family Viscaceae have small, inconspicuous flowers while the mistletoes in the Loranthaceae family have large, colourful flowers (Hawksworth and Wiens 1970; Hawksworth and Wiens 1996; Geils and Hawksworth 2002).

One of the most important mistletoe genera to North America forestry is *Arceuthobium*. Dwarf mistletoes (*Arceuthobium* spp., Viscaceae) are flowering plants that parasitize members of Pinaceae and Cupressaceae family. These parasites are found in North America, Central America, Europe (Spain), Asia, and Africa (Hawksworth and Wiens 1996; Mathiasen 1996, Geils and Hawksworth 2002). The genus consists of about 42 species. In the Old World, eight species of *Arceuthobium* are found from Spain and Morocco to the Himalayas of southwestern China, with outlying species found in East

Africa. In the New World, the genus is represented by 34 taxa found mainly in Central America, Mexico, and western North America (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). Twenty-eight species occur in the United States and Canada. Of the five species of dwarf mistletoe found in Canada, four occur in British Columbia (Geils and Hawksworth 2002).

1.2.2 Characteristics of *Arceuthobium*

The dwarf mistletoes are dioecious, epiphytic obligate parasites with segmented stems and greatly reduced leaves. Obligate parasites are incapable of establishing and/or developing independently from their host plant (Hawksworth and Wiens 1996; Watson 2001). Parasitic plants can be divided into hemi- or homoparasites depending on the nutrients that they require from their hosts. Homoparasites lack chlorophyll and are unable to synthesize carbon sources. Dwarf mistletoes are hemiparasites that produce their own chlorophyll and can photosynthesize but obtain water, minerals, and some photosynthates from their hosts (Watson 2001).

Male flowers are 3-merous with sessile stamens. Female flowers are 2-merous and consist of a single, inferior ovary with a basal placentation (Hawksworth and Wiens 1996). Dwarf mistletoes have no true ovules. Their embryo sac originates from tissue at the ovary, meaning that the seed lacks a testa or seed coat. The seed is an embryo embedded in chlorophyllous endosperm surrounded in viscin (Knutson 1983; Hawksworth and Wiens 1996).

The genus *Arceuthobium* is considered to be the most specialized in the Viscaceae with respect to the parasitic habit. Some of these more specialized features include:

1. Explosive fruit (Figure 1. 1),
2. Small flowers (Figure 1.2),
3. An endophytic system that includes cortical strands and sinkers (Figure 1. 4),
4. Induction of witches' brooms on their hosts (Figure 1.3), and
5. Reduced leaves called scales or vesticular leaves (Hawksworth and Wiens 1996).
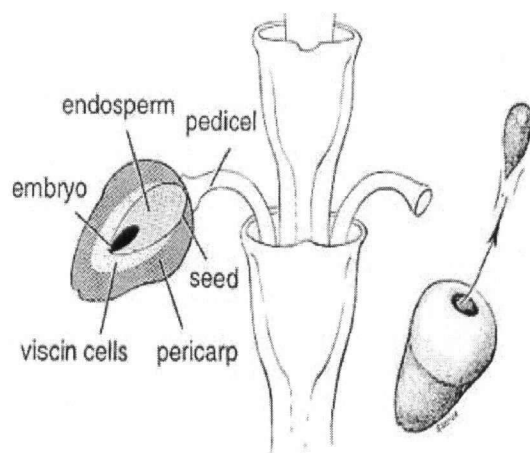
Figure 1-1 Dwarf mistletoe berry and seed. Note seed dispersal from the dwarf mistletoe berry.
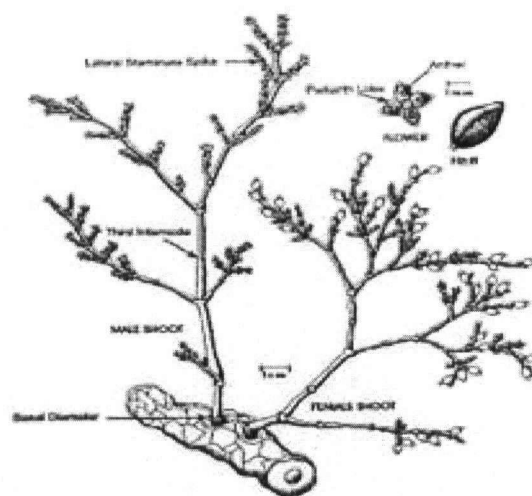
Figure 1-2 Dwarf mistletoe shoot with male and female flowers (Dwarf mistletoe plants are dioecious therefore each shoot represents one plant).

Figure 1-3 Western hemlock stand infected with dwarf
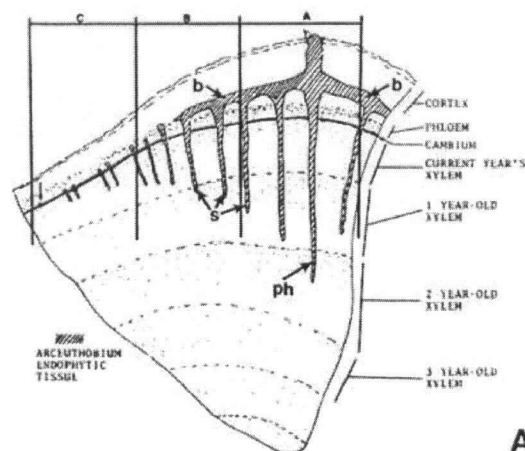
Figure 1-4 Endophytic system of a dwarf mistletoe plant.

- **Pictures are from Hawksworth and Wiens 1996 (Mistletoe Centre)-Permission to use pictures from Dr. B. Geils 2006.**

Arceuthobium is considered a taxonomically difficult genus due to the extreme morphological reductions associated within this parasitic habitat and the general morphological similarities among the species (Hawksworth and Wiens 1996). Many of

the taxonomic features found in other plants, such as leaves and trichomes, are absent or reduced in *Arceuthobium*. Flowers are small and similar in form to other plants. Life cycles and various morphological, physiological, and biochemical features have been used to determine taxonomy in *Arceuthobium* classification of the genus (Hawksworth and Wiens 1996; Wass and Mathiasen 2003).

1.2.3 Symptoms and Signs of *Arceuthobium*

The first visual symptom of dwarf mistletoe infection is a localized swelling of branches or stems of the host (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). As the infection develops, the swelling enlarges. After a few years flower-bearing shoots begin to emerge. Fruit matures in the fall, 12–16 months after anthesis (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). Shoots are generally short lived and leave "basal cups" when they are shed. On dormant or older infections such basal cups may be the only direct indication of mistletoe infection on the branch (Hawksworth and Wiens 1996; Smith 1977; Geils and Hawksworth 2002; Hennon et al. 2001). Abnormal enlarged, swollen, or crooked branches usually occur even when brooming is inconspicuous on older trees (Hawksworth and Wiens 1996; Geils and Hawksworth 2002).

Typical brooms of dwarf mistletoes are spherical balls or flat fans of growth (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). In heavily infected stands, severe brooming results in top kill, stunting, and eventually host mortality (Figure 1.3) (Unger 1992; Hawksworth and Wiens 1996; Geils and Hawksworth 2002). In young stands, infection centres will occur around the larger and older infected trees (Geils and Hawksworth 2002).

1.2.4 Biology and Ecology of *Arceuthobium*

The dwarf mistletoe initiates the disease by penetrating the host tissue and producing hormones such as cytokinins (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). These hormones increase the translocation of nutrients to the dwarf mistletoe at the expense of the host's uninfected parts. The increase of hormones in the infected areas result in an increased number of branches in the dwarf mistletoe infected areas (Johnson 1989; Hawksworth and Wiens 1996). The high transpiration rate of dwarf mistletoe

shoots can cause a disproportionately higher rate of water and nutrient movement to infected sites (Alosi 1979; Johnson 1989; Hawksworth and Wiens 1996). These infected areas act as nutrient (sugar, amino acid, minerals, and amines) and water sinks for the host resulting in loss of tree vigour (Alosi 1979; Hawksworth and Wiens 1996; Pennings and Callaway 2002). Secondary pathogens, or insects such as bark beetles, often become established in weakened trees, hastening death (Hawksworth and Wiens 1996; Alosi and Barkley 1997). The overall energy drain on the host is relatively small with one dwarf mistletoe broom. However, large numbers of brooms result in a detectable decrease in host height and diameter growth (Hawksworth and Weins 1996; Smith 1977). Dwarf mistletoe infections can also reduce the quality and quantity of tree seed (Smith 1977; Hawksworth and Weins 1996).

The effects of dwarf mistletoe on its host are reduced growth rates, vigor, and wood quality. Reduced wood quality is a result of decreases in wood strength, abnormal grain, and spongy wood texture (Unger 1992; B.C. Ministry of Forests 1995; Hawksworth and Weins 1996; Hennon et al. 2001). Individual small trees and mature trees that are heavily infected can be killed, and in time the growth of living trees can completely stagnate (Allen et al. 1996; Geils and Hawksworth 2002). Infected wood is weaker and has poor pulping qualities. Infections can occur in the main stem of trees, some of which originate in side branches and grow into the bole (B.C. Ministry of Forests 1995; Geils and Hawksworth 2002). These bole infections result in a burl-like structure, distorting trunks and resulting in large knots in the wood (Hawksworth and Weins 1996; Hennon et al. 2001; B.C. Ministry of Forests 2002). In species such as pines, heavily infected dwarf mistletoe trees result in large proportions of dead foliage and branches. Dwarf mistletoe infected trees are also very combustible; making them serious fire hazards (Alosi and Barkley 1997). In recreational areas, large brooms can create a hazard, since these infected branches may break and fall. Severely infected stands increase opportunities for outbreaks of insects, disease, and fire (Hennon et al. 2001; Geils and Hawksworth 2002).

Special management strategies and silviculture treatments for infected stands are required when the objectives are to maintain and enhance wildlife habitat—the focus is on maintaining snags, brooms, birds, and mammals (Tinnin et al. 1982; Hawksworth and Wiens 1996; Tinnin and Forbes 1999; Geils and Hawksworth 2002).

Arceuthobium-infected stands do not provide a large incentive for birds or mammals to visit for pollination or seed dispersal compared with other mistletoes. *Arceuthobium*-infected stands do offer forage, resting, nesting, protection, special sites, and desirable stand structures for numerous wildlife species (Tinnin et al. 1982; Tinnin and Forbes 1999; Geils and Hawksworth 2002). Animals such as squirrels are known to selectively feed on the swollen nutritious tissues such as hemlock dwarf mistletoe infections (Hennon et al. 2001; Geils and Hawksworth 2002).

1.2.4.1 Life Cycle of *Arceuthobium*

The spread of dwarf mistletoe in a stand occurs via explosively discharged seeds. The fruit is a berry that contains a small (about 3 mm long) seed. When the berry is ripe, hydrostatic pressure builds up in the fruit until it ruptures at the base, forcibly discharging the seed for a distance of 6 to 15 m (Smith 1977; Allen et al. 1996; Hawksworth and Weins 1996; Geils and Hawksworth 2002). Seed interception rates depend on a number of factors such as stand structure, tree composition, the relative size of infected trees, and the location of mistletoe seed producing infections on such trees (Smith 1977; Smith 1985; Mathiasen 1996).

Seeds are dispersed in late summer to early November for most Arceuthobium species (Smith 1985; Hawksworth and Wiens 1996). Seeds are covered in a sticky viscin, which enables them to adhere to the foliage or branches of other trees. The chlorophyllous endosperm helps maintain the embryo and permits growth of the hypocotyls (Kuijt 1960; Knutson 1983; Geils and Hawksworth 2002). The concentration of chlorophyll in dwarf mistletoe seeds of *A. tsugense* and *A. campylopodium* is similar to that found in the dwarf mistletoe shoots (Knutson 1983).

During periods of rain following seed dispersal, the seed and viscin absorb water, swell, and become slippery (Smith 1977; Hawksworth and Wiens 1996; Geils and Hawksworth 2002). The slippery viscin allows the dwarf mistletoe seed to slide to the base of the needle or twig where it becomes glued and over-winters (Hawksworth and Wiens 1996; Brandt et al. 2003). Seeds on pendant needles may slide off and fall to the ground or lower needles. In the spring, dwarf mistletoe seeds germinate and produce short radicles, which form holdfasts (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). The chlorophyllous endosperm provides nutrients to dwarf mistletoe

seed until an endophytic system becomes established (Knutson 1983; Hawksworth and Wiens 1996).

Successful infection by dwarf mistletoe requires the penetration of the host cortex. The elongating radicle of the germinating seed grows along the surface of the twig until it meets an obstruction or break in the bark, usually at the base of the needle (Kuijt 1960; Brandt et al. 2003). The tip of the radicle then forms a holdfast from which an infection peg penetrates the bark tissue, crushing the host tissue (Hunt et al. 1996; Hennon et al. 2001). Hunt et al. (1996) noted that mechanical forces are very important for penetration. The role of the enzymes in penetration by dwarf mistletoes is uncertain. Penetration is largely restricted to stem tissues usually less than 5 years old (Hawksworth and Wiens 1996; Geils and Hawksworth 2002; Brandt et al. 2003).

Once the host periderm has been penetrated, infection of the host branch becomes established (Kuijt 1960; Hawksworth and Wiens 1996; Hunt et al. 1996; Geils and Hawksworth 2002). The infection peg penetrates the host's phellem and enters the cortex; a root-like structure known as the endophytic system ramifies throughout the bark (Kuijt 1960; Hunt et al. 1996; Hawksworth and Wiens 1996; Brandt et al. 2003). The buds of dwarf mistletoe push through the cork cambium and develop into shoots (Hawksworth and Wiens 1996; Hunt et al. 1996; Geils and Hawksworth 2002). The endophytic system is a perennial structure that produces aerial shoots continuously (Hawksworth and Wiens 1996). Shoots emerge 2–5 years after inoculation in the host branch (Kuijt 1960; Smith 1977; Hawksworth and Wiens 1996). After shoots emerge from the host branch, it can take two or more years before flowers are produced (Hawksworth and Wiens 1996). Dwarf mistletoe plants are dioecious with most shoots producing two or more successive crops of flowers (Hawksworth and Wiens 1996). Wind or insect pollination of the female dwarf mistletoe flowers occurs from July to September for most dwarf mistletoe species (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). Pollen dispersal can occur up to 150 m away from the male flowers (Hawksworth and Wiens 1996). The fruits mature in fall of the year following pollination (Hawksworth and Wiens 1996; Hennon et al. 2001; Brandt et al. 2003) (Figure 1.5) with the exception of *A. pusillum* that produces fruit in the same year as flowering.

Dwarf mistletoe infections that live on the lower branches will eventually be shaded, lose all their shoots, and become dormant (Richardson and van der Kamp 1972; van der Kamp 1998). Smith (1977) has suggested that dwarf mistletoes can live in this dormant state indefinitely. These dormant dwarf mistletoe infections can be reactivated if, as result of selective logging, light levels to the infection increase (Richardson and van der Kamp 1972; Mathiasen 1996; van der Kamp 1998; Shaw and Weiss 2000).

1.2.4.2 Biology of *Arceuthobium* spp.

Dwarf mistletoe plants are commonly described as leafless, but leaves have been reduced to a pair of minute scales (vestigial leaves) that vary in shape and size (Hawksworth and Wiens 1970; Hawksworth and Wiens 1996). The simple small square and round vestigial leaves are initiated at the periphery of the dwarf mistletoe shoot apex (Hawksworth and Wiens 1996; Hawksworth et al. 2002). The colour of the shoots can vary from yellow to brown or olive green in many shades (Hawksworth and Wiens 1996; Hawksworth et al. 2002). Several different branching patterns in dwarf mistletoe shoots occur such as decussate (crossed at right angles) branching and shoots with decussate phyllotaxis (Kuijt 1960; Hawksworth and Wiens 1970; Hawksworth and Wiens 1996).

The vestigial leaves have an epidermis with guard cells. No trichomes are present and a thick cuticular layer of wax covers the epidermis (Hawksworth and Wiens 1970; Hawksworth and Wiens 1996). Sub-epidermal layers of the dwarf mistletoe leaves consist of sclerified parenchyma cells with tracheary elements and ground tissue mainly made up of parenchyma, and chlorenchyma layer (Hawksworth and Wiens 1970; Hawksworth and Wiens 1996). No differentiation of the mesophyll into palisade and spongy tissue occurs in dwarf mistletoe species (Hawksworth and Weins 1996).

The shoots of dwarf mistletoes consist of the stems and attached vestigial leaves. The boundaries between the vestigial leaves and stems are not evident (Hawksworth and Weins 1996). Leaf modifications for dwarf mistletoes include (1) low external surface-to-volume ratio, (2) sunken guard cells, (3) small lower stomata, (4) lack of intercellular space in the mesophyll, and (5) thick cuticular layer indicating that dwarf mistletoe species can survive xerophytic situations (Hawksworth and Wiens 1996). The shoots bear the flowers and fruits, but synthesize little food (Hawksworth and Wiens 1996).

Shoots develop into male and female dwarf mistletoe plants immediately before the shoots form flowers. Each *Arceuthobium* species produces flowers annually on mature dwarf mistletoe shoots (Kuijt 1960; Hawksworth and Wiens 1996). Flowering generally lasts 4–6 weeks and pollen is dispersed in the first 2–3 weeks (Hawksworth and Weins 1996; Geils and Hawksworth 2002).

The endophytic system of dwarf mistletoe differs from typical angiosperm roots in that it lacks a root cap, pericycle, endodermis, and continuous phloem and xylem (Cohen 1954; Hawksworth and Wiens 1996). A localized infection develops haustoria (cortical) strands, which extend longitudinally and tangentially through the host cortex and the outer host phloem (Cohen 1954; Alosi and Calvin 1984). Once the haustorial strands grow towards the host's vascular cambium and contact it, sinkers are initiated (Cohen 1954; Alosi and Calvin 1984; Hawksworth and Wiens 1996). These sinkers have radially developed growth and extend from the haustorial strands in the secondary vascular system of the tree branch. Sinkers are not considered distinct organs of the haustorial system but refer to the radial portion of the haustorium (Alsoi and Calvin 1984; Geils and Hawksworth 2002).

Sinker tissue is almost always associated with the rays. When young sinkers develop in a region where no ray cells exist, new ray cells quickly develop in association with the sinker cells. The "infection ray" develops as result of radially orientated sinker cell surround by the host ray cells (Cohen 1954; Alsoi and Calvin 1984; Hawksworth and Wiens 1996). Infection rays consist of host ray parenchyma, host albuminous cells, sinker ground parenchyma, and sinker sheath cells (Alsoi and Calvin 1984; Hawksworth and Wiens 1996).

Arceuthobium sinkers are thought to be responsible for carbohydrates that are absorbed as simple sugars and moved to dwarf mistletoe shoots by turgour pressure (Alosi and Calvin 1985). Srivastava and Esau (1961) proposed that sinkers penetrate the cortex of the host and connections to the cambium and the secondary phloem allowing mistletoe plant to absorb nutrients (Alosi and Calvin 1984; Hawksworth and Wiens 1996; Geils and Hawksworth 2002). The transfer of water, minerals, and photosynthates and possibly organic compounds originating from the host to the dwarf mistletoe occurs in a

common apoplast (Alosi and Calvin 1984). Alosi and Calvin (1985) suggest that host cells leak nutrients into the common apoplast and the sinker cells absorb these nutrients.

Localized or anisophasic infections are endophytic systems of some dwarf mistletoe species and are generally restricted to within or near the swollen portion of the host. Anisophasic endophytic growth keeps pace with the cambium growth of the host's branch (Kuitj 1960; Hawksworth and Wiens 1996; Geils and Hawksworth 2002). The production of shoots, which usually occurs between 2 and 5 years, is localized within the swollen portions of the branch (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). These brooms are typically fusiform (Kuitj 1960; Smith and Wass 1979; Hawksworth and Wiens 1996). Some examples of dwarf mistletoe species that have localized infections: *A. tsugense* on *Tsuga heterophylla*; *A. americanum* on *Picea glauca, Pinus jeffreyi and Picea engelmanii*; *A. laricis* on *Larix occidentalis*; *Arceuthobium douglasii* on *Abies* spp.; and *A. campylopodum* on *Abies, Larix*, and *Picea* spp. (Kuijt 1960; Hawksworth and Wiens 1996; Geils and Hawksworth 2002).

Systematic infections or isophasic infections result in endophytic system growth keeping pace with the apical and cambium growth with the apices of the host (Kuijt 1960; Hawksworth and Wiens 1996). In isophasic infections, the endophytic system spreads out through the host tissue or is concentrated at branch nodes of dwarf mistletoe infected branches that initiate from single seed infection (Kuijt 1960; Alosi and Calvin 1984; Hawksworth and Wiens 1996). Shoot production from these swelling usually initiated on 2–4 old year branch nodes (B. van der Kamp, pers. comm., 2005). Typical isophasic infections lack swelling and are usually more elongated and pendulous than anisophasic infections (Kuijt 1960).

One of the most distinguishing features of an isophasic broom is the regular shoot emergence pattern that is repeated annually (Kuijt 1960). Fewer dwarf mistletoes species can induce isophasic infections than anisophasic infections (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). Examples of dwarf mistletoes that produce isophasic infections include *A. americanum* on *Pinus contorta, A. douglasii* on *Pseudotsuga menziesii,* and *Arceuthobium pusillum* on *Picea* spp. (Kuijt 1960). Isophasic infections are thought to be more evolutionary advanced, since this broom type is more efficient at infection than localized infections (Hawksworth and Wiens 1996). Kuijt (1960) noted that

anisophasic brooms could become sterile and produce no fruit. Isophasic brooms usually do not become sterile, since the shoots are in a good position for photosynthesis, pollination, and seed dispersal. In general isophasic brooms can produce more productive shoots than anisophasic brooms (Kuijt 1960).

Some dwarf mistletoe swellings combine both types of brooming. These cases are rare and may be phenotypic responses to some dwarf mistletoes. Such combinations of isophasic and anisophasic brooms have been described in *A. globosum* and *A. occidentale* (Hawksworth and Wiens 1996; Geils and Hawksworth 2002).

1.2.5   Host Specificity of *Arceuthobium* spp.

Dwarf mistletoes exhibit varying degrees of host specificity. Typically a particular species of dwarf mistletoe will proliferate rapidly on some hosts, and less so on other hosts while some tree species are immune. All are found on conifers; hardwoods are always immune (Hawksworth and Wiens 1996). The hosts for particular dwarf mistletoe are commonly classified into five natural susceptibility classes based on the percentage infected of trees within 6 m of infected major host that is dispersing dwarf mistletoe seed. Principal hosts show 90–100% infection, secondary hosts show 50–90% infection, occasional hosts show 5–50% infection, rare hosts show 1–5% infection, while immune hosts show no infection (Hawksworth and Wiens 1972; Hawksworth and Weins 1996; B.C. Ministry of Forests and Range 2002; Geils and Hawksworth 2002).

In the New World, the principal hosts of dwarf mistletoe include *Abies, Larix, Picea, Pinus, Pseudotsuga, and Tsuga*. In the Old World, principal hosts are *Abies, Keteleeria, Juniperus, Picea,* and *Pinus*. Host specificity of dwarf mistletoes can vary from being very specific such as *A. abietinum* Engelmann ex Munz f. sp. *magnifica*, Hawksworth & Wiens, which only infects *Abies magnifica* to quite general, such as *A. globosum*, which has up to 12 different Pinus principal hosts (Hawksworth and Wiens 1970; Hawksworth and Wiens 1996).

In Canada, dwarf mistletoes seriously affect the following tree species: western hemlock, lodgepole pine, jack pine (*Pinus banksiana* Lamb), western larch, and black spruce (*Picea mariana* [Mill.] BSP) (Unger 1992; Hawksworth and Wiens 1996). In the western United States, dwarf mistletoes seriously damage trees such as ponderosa pine

(*Pinus ponderosa* P. Laws ex C. Laws), lodgepole pine, Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.), Douglas-fir, California red fir (*Abies magnifica* Doug. ex D. Don), white fir (*Abies 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 infections in North America (Hawksworth and Wiens 1996).

In North America, 85 taxa of Pinaceae are naturally infected with *Arceuthobium*. Artificial inoculation trials with tree species not found in the natural range of specific species of *Arceuthobium* have identified a number of additional potential hosts (Hawksworth and Weins 1996; Geils and Hawksworth 2002). Such tests are important due to economic losses that can occur when planting new tree species outside their natural range. Existing *Arceuthobium* can severely infect trees in a new planting area. Prior knowledge of the susceptibility of the tree species to indigenous dwarf mistletoe species found at the planting site can help prevent timber loss (Hawksworth and Weins 1996; Geils and Hawksworth 2002).

1.2.6 Spread and Intensification of *Arceuthobium* spp.

Tree density, which directly influences the canopy structure of a forest, also influences the spread of mistletoe. Generally, dwarf mistletoe spreads more rapidly in open canopy structures. In areas with few trees and with canopies located far apart (>15 m), mistletoe spread can be reduced because the mistletoe seed cannot reach neighboring crowns (Smith 1977; B.C. Ministry of Forests 1983; Wass and Mathiasen 2003; Wass 2006). Other factors that influence mistletoe seed spread include host tree distances, topography, canopy height, canopy structure, and non-host trees. When canopies close, they screen seeds from reaching beyond the trees immediately surrounding the infected tree. Upward spread of dwarf mistletoe averages about 40 cm/yr in less dense stands (Smith 1977; B.C. Ministry of Forests 1983; Mathiasen 1996; Shaw and Weiss 2000). Mistletoe seed germination, establishment, and reproduction are determined by light intensities, weather, genetics, and host susceptibility (Richardson and van der Kamp 1972; Knutson 1983; van der Kamp 1998; Geils and Hawksworth 2002).

Dwarf mistletoe is spread by the explosive discharge of the seed within the crown to nearby hosts. Spread is most rapid in forests with multiple size classes of host trees (Hawksworth and Wiens 1996; Mathiasen 1996; Geils and Hawksworth 2002). Smaller under storey trees are continuously exposed to dwarf mistletoe seed from the infected over storey trees. In even-aged stands, spread of dwarf mistletoe is much slower than in multi-aged forests due to the close canopies (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). In dense even-aged stands, lateral spread is probably less than 50 cm/yr, since the dense foliage reduces light intensity that is necessary for seed production and intercepts the discharged seed (Smith 1977; Mathiasen 1996; Shaw and Weiss 2000). In open-grown trees less than 15 m tall, dwarf mistletoe spreads faster because increased light favours seed production, and the seeds are discharged for greater distances due to reduced blockage from other trees (B.C. Ministry of Forests 1983; Smith 1985; Mathiasen 1996).

Dwarf mistletoe seed dispersal is responsible for spread and intensification of dwarf mistletoe (Smith 1977; B.C. Ministry of Forests 1983; Hawksworth and Wiens 1996; Mathiasen 1996). The terms "spread" and "intensification" are commonly used to describe changes in mistletoe occurrence and severity levels. When a tree progresses from an initial DMR of 0 to a higher number it means that the dwarf mistletoe has infected the tree. When an increase in a DMR is greater than 0 (DMR changes from a 2 to a 5, for example) it means that the infection has "intensified" in severity (Smith 1977; B.C. Ministry of Forests 1983; Hawksworth and Weins 1996; B.C. Ministry of Forests and Range 2002). On heavily infected trees thousands of seeds can be dispersed, but it has been estimated that only 5% of the seeds hit the target host and 1–2% establish infections (Mathiasen 1996). Species with dense crowns such as true firs (*Abies* spp.) block dispersal of seed, slowing the spread of dwarf mistletoe (Mathiasen 1996). Species with open crowns may allow more seed to escape, increasing the likelihood that seeds will be intercepted by a nearby host crown. Seeds are screened or deflected by closed canopies (Mathiasen 1996). Closed canopies can also intercept dwarf mistletoe seed from beyond the infected trees and protect the inner canopy trees, which slow the spread of dwarf mistletoe seed. Distance is important between healthy and diseased trees (e.g., the

closer a tree is to an infected tree, the higher the probability of dwarf mistletoe spread) (Smith 1977; B.C. Ministry of Forests 1983; Mathiasen 1996).

Birds and mammals disperse a small percentage of hemlock mistletoe seeds and are responsible for the long-range dispersal of dwarf mistletoe (Geils and Hawksworth 2002). In dense stands, dwarf mistletoe infections in the middle and upper crown region are the most damaging (Smith 1977; B.C. Ministry of Forests 1983; Shaw and Weiss 2000). The occurrence of mistletoe in the lower crown region is of less of concern, since spread is small and the infection is shaded out (Richardson and van der Kamp 1972; van der Kamp 1998).

Dwarf mistletoe infestations can result in a small scale gap formation occurring in mature forests. This means that heavily infected areas of mature trees in a forest can form gaps by tree death (Edmonds et al. 2000; Geils and Hawksworth 2002). Gap formation allows vegetation different from the normal mature stands to form, such as different tree species or seedlings that are less susceptible to dwarf mistletoe (Edmonds et al. 2000; Dorner and Wong 2003). Infection of susceptible tree species in adjacent regenerating plantations can result from gap formation (Smith 1977; B.C. Ministry of Forests 1983). Gap formation can also enhance the dispersal mechanism both horizontally and vertically by allowing the dwarf mistletoe to penetrate into most areas where one or few trees have died or blown over (Smith 1977; B.C. Ministry of Forests 1983; Trummer et al. 1998). Regeneration that develops after complete harvest or stand destruction is seldom infested. Disturbance or stand replacement events such as fires, windstorms, and other severe disease outbreaks can reduce dwarf mistletoe (Geils and Hawksworth 2002; Muir 2003).

Forests infested with dwarf mistletoes have different canopies than similar non-infested forests (Geils and Hawksworth 2002). The more branches are infected with dwarf mistletoe, the more brooms form, until a tree may become one large mass of dwarf mistletoe brooms (Hawksworth and Wiens 1996). As the dwarf mistletoe infection spreads through the forests, the whole canopy structure becomes dominated with trees with large witches' brooms, spike tops, and dead trees (Geils and Hawksworth 2002). The stand can stay in this state permanently. Individual trees may die, but the structure remains unchanged until a stand-destroying disturbance resets the system (B. van der Kamp, pers. comm., 2005) (Figure 1.3). These changes in the forest canopy may take

many years (Richardson and van der Kamp 1972; Mathiasen 1996; van der Kamp 1998; Geils and Hawksworth 2002).

Often, dwarf mistletoe infection is patchy within a stand, with discrete infection centres. Over time, many of these centres may coalesce to form infections for many hectares (Tinnin et al. 1982). These forests consist of a mixture of dead trees, heavily broomed trees, lightly infected younger trees, and some trees that escape infection. As trees die directly or indirectly as result of parasitism by the dwarf mistletoe, many snags are created within infested stands to the point where it no longer resembles the canopy structure of a healthy stand (Tinnin et al. 1982). In mixed-species forests, the effects of dwarf mistletoe infection on canopy structure will be less pronounced, since the less susceptible or immune species will reduce the spread to the susceptible species (B.C. Ministry of Forests 1983; Hawksworth and Wiens 1996; Geils and Hawksworth 2002).

Where stands are heavily infected without the sanitizing effects of fire or cutting practices, which reduces the mistletoe, habitat quality can decline over the long term (Tinnin et al. 1982; Geils and Hawksworth 2002).

1.3 Management Options for *Arceuthobium* spp.

1.3.1 Silvicultural Control Management

In British Columbia, stands infected with dwarf mistletoe are most effectively controlled through silviculture practices. Silviculture management of dwarf mistletoe infected forests is determined by different factors such as the intensity of the site utilization (production of timber and fiber or recreation, wildlife, and other ecosystem values); infection percentage; structure; stand density; tree distribution; site; proportion of host and non-host trees; severity of the infection; and number of years to harvest (B.C. Ministry of Forests 1983; Hawksworth and Weins 1996; Geils and Hawksworth 2002). In stands where timber production or intensive recreation uses are major considerations, control of dwarf mistletoe may be necessary to meet management objectives. In other areas where wildlife habitat or other ecological uses are prime considerations, dwarf mistletoe populations are often not managed (Hennon et al. 2001).

Dwarf mistletoe is extremely variable in incidence and severity. To quantify the severity of a dwarf mistletoe infection on a tree in British Columbia, the Ministry of

Forests uses the Hawksworth six-class system. The expected effects of dwarf mistletoe on tree growth and survival have been strongly correlated with infection severity or the amount of the crown colonized with the mistletoe plants (B.C. Ministry of Forests 1983; B.C. Ministry of Forests 1995; Hawksworth and Wiens 1996). The Hawksworth rating system is based on the number of infections found in the crown of the tree, and divides the crown into thirds. Each third of the crown is rated based on the number of infections for each crown area. A rating of 0 is given for no visible infections, 1 for light infection ($\leq$50% of the total number of branches in the third of the tree is infected), and 2 for heavy infection (>50% of the total number of branches in the third of the tree is infected). The rating for each third is summed for a tree. On a tree or stand basis, light infection is a rating of 1–2, moderate is 3–4, and severe is 5–6 (B.C. Ministry of Forests 1983; Hawksworth and Wiens 1996; B.C. Ministry of Forests 2002; Geils and Hawksworth 2002). In some tree species the dwarf mistletoe can infect the main stem. In cases of minor stem infection (25% of the stem circumference affected), rating N is given. For major stem infections (>25% of the stem circumference affect), the rating of M is given. For a stand, the rating is calculated as the average rating of all infected trees and the incidence is the percentage of susceptible trees infected by dwarf mistletoe. A dwarf mistletoe rating can be determined for a tree, plot, or stand. By determining average dwarf mistletoe rating (DMR) and the composition of the host trees in a stand, important forest management decisions can be made such as when to thin or harvest infected stands (B.C. Ministry of Forests 1995; B.C. Ministry of Forests and Range 2002).

The most effective method of control for dwarf mistletoe is clear cutting the infested stand. Clearcut harvesting coupled with eradication of all infected trees reduces the spread and intensity of the dwarf mistletoe (B.C. Ministry of Forests 1983; B.C. Ministry of Forests 1995; Geils and Hawksworth 2002). To prevent or reduce the spread of dwarf mistletoe in traditional silvicultural systems, a number of preventative measures can be used:

1. Design cut block boundaries in non-infested stands and non-susceptible stands or takes advantage of natural or constructed openings such as roads, stream openings, and meadows that prevent reinvasion from adjacent infested stands.

2. Remove all infected trees before an area is planted or naturally regenerated with susceptible species.

3. Avoid leaving single trees or small clumps of trees infected with dwarf mistletoe throughout a harvested area. The increase in light can activate dormant mistletoe infections on the overstorey trees and spread to a regenerating stand.

4. If infected trees are to be left on the boundary, avoid leaving fringes or narrow strips. Maintain a dense block of trees and leave a relatively uniform, abrupt margin. Where spread and infection of dwarf mistletoe occur to new regenerating trees, remove residual trees at the next silviculture treatment.

5. On sites where non-susceptible tree species occur that are able to compete susceptible species, plant block borders with a mixture of non-susceptible tree species. On most sites, rapid regeneration of susceptible tree species occurs and this treatment will not work. This is often the case on sites that are dominated by western hemlock.

6. Design cut blocks within infested stands to create a high ratio of area to perimeter and to minimize the length of infested border (avoid long narrow blocks and units <8 ha) (B.C. Ministry of Forests 2005).

The B.C. Ministry of Forests has recently implemented variable retention harvest systems as an alternative to clear cutting (B.C Ministry of Forests 1995; Mitchell and Beese 2002; Vyse 2004). The concept of variable retention or partial cutting systems was to develop and adopt guidelines in British Columbia that will achieve public acceptability and to protect the natural forest ecosystems (Mihai 2004; Sheppard 2004). In the 1990s the B.C. Ministry of Forests developed a research and extension program to examine ecological, economic, and social issues surrounding the use of alternative silvicultural systems such as variable retention (Vyse 2004). Variable retention is a silvicultural system that conserves the biological diversity, ecosystem function, and structural heterogeneity in managed forests (Mitchell and Beese 2002). Ideally this system emulates natural stand disturbance regimes and drives forest succession in a manner similar to that in natural and unmanaged forests (Beese 2004).

The riparian forest areas with moist soils support plant communities that are distinct from those of the surrounding uplands (B.C. Ministry of Forests 1995; Banner and McKenzie 2000). Riparian ecosystems can vary in width from just a few meters, next to a small stream with banks, to more than 100 m near large rivers (B.C. Ministry of Forests 1995). Healthy riparian areas are recognized as centres of high biodiversity, and include three critical habitat components for wildlife: water, cover, and food. Riparian ecosystems around streams and rivers help protect habitats for fish and other aquatic species (Banner and McKenzie 2000). In British Columbia, riparian areas represent about 10% of the provincial land base. The Forest Practices Code (FPC) acknowledged the importance of riparian ecosystems. The Riparian Management Area Guidebook (B.C. Ministry of Forests 2002) uses wetland classification systems to set widths for protective riparian management areas around wetlands. In some cases harvesting is restricted within 30 m of the waterway. In some situations variable retention is used within riparian forests (Richardson 2003).

In variable retention silvicultural systems and riparian forests, the options to control dwarf mistletoe are limited compared with clear cutting. Control treatments for dwarf mistletoe on variable retention sites may not be available due to harvest site conditions (e.g., riparian site, the type of variable retention used for the site, and tree species that dominate the site) (Muir 2003; Shamoun et al. 2003; Muir et al. 2004). .

Muir et al. (2004) suggests a number of methods for the control of dwarf mistletoe found in a variable retention silvicultural system:

1. Avoid or minimize residual trees in the boundaries and in dispersed or aggregate reserves.

2. Plan to plant any susceptible host trees at least 10 m from any residual trees.

3. Remove or kill any infected residual trees that are 3 m or taller adjacent to harvest site.

4. When possible establish dense stands to slow the spread of dwarf mistletoe and to accelerate shading out of infections.

5. Plant non-susceptible species or genetically resistant trees (both of these options may not be feasible because certain sites may only grow susceptible species and

genetically resistant planting stock is not available (B.C. Forestry Practices Code
1995, Ministry of Forest 2005).

Bloomberg et al. (1980) and Bloomberg and Smith (1982) developed one of the first
mistletoe models for predicting spread and intensification of dwarf mistletoe. ESSA
Technologies Ltd. was developing models that assist foresters and others to determine
tolerable or acceptable levels of dwarf mistletoe damage under various environmental
conditions or forest practices (Robinson et al. 2000; Robinson et al. 2002). This model
will be linked to the B.C. Ministry of Forests Tree and Stand Simulator (TASS) to
determine growth losses from dwarf mistletoe (Muir et al. 2004).

Tree growth and dwarf mistletoe models can be useful tools for resource managers in
simulating yields in infected stands. Yields for a stand can be predicted under different
management regimes and compared with sites with no treatments. By comparing outputs
and economic analysis of control costs, the manager can choose the best treatment for
each infested stand and priorize treatment (Robinson et al. 2002; Muir and Moody 2002;
Muir et al. 2004; Mihai 2004;). Three stands infected with dwarf mistletoe in British
Columbia have been inputted into model for testing. More historic dwarf mistletoe stand
data is required for the model to project accurate scenarios of dwarf mistletoe growth
losses in B.C. (Robinson et al. 2000; B. van der Kamp, pers. comm., 2005).

1.3.2   Chemical Control

Chemical control of dwarf mistletoe using selective herbicides has been ineffective. It
is difficult to find a chemical that is easy to apply and that will kill the mistletoe without
being toxic to the host and to other non-target species. The alternate method of control of
dwarf mistletoe infection is to cause the abscission of the shoots, thereby reducing and
delaying spread and intensification (Shamoun and DeWald 2002; Shamoun et al. 2003).

The first selective herbicides screened to control dwarf mistletoe included a wide
range of herbicides, especially various salts and esters of 2, 4-D or 2, 4, 5-T. Over 60
different chemicals were tested with various formulations of 2, 4,-D or 2, 4, 5-T. None
have been identified that would kill the dwarf mistletoe without killing or harming the
host (Gill 1956; Quick 1963, 1964; Scharpf 1978). To reduce the toxic effects on the host,
lower rates of the herbicides were applied to the swellings. These lower rates did not kill

the endophytic system of the dwarf mistletoe plants and after several years resulted in the formation of new shoots. The most promising herbicide screened was isococtyl esters of 2, 4, 5-T developed by Quick in 1964. Despite its promise, 2, 4, 5-T found little acceptance as an operational management tool for the control of dwarf mistletoe and was banned because of its environmental effects (Parkes and Hoffman 1991; Hawksworth and Wiens 1996; Shamoun and DeWald. 2002; Shamoun et al. 2003).

Herbicides and growth regulators were screened on dwarf mistletoe from 1970 to the 1990s. These chemicals included 2-4-D (Dacamine), MCPA, 2-4DB (Butyrac), oxyfluofen (Goal), MCPM (Thistrol), silvex (Weedone), Emulsamine, GA-41065 (Prime), and ethephon (Florel) (Hawksworth and Weins 1996; Shamoun and DeWald 2002; Shamoun et al. 2003). Most of these herbicides resulted in high mortality of dwarf mistletoe shoots and minimal host damage. None, however, affected the endophytic system of the dwarf mistletoe (Hawksworth and Wiens 1996; Shamoun and DeWald 2002; Shamoun et al. 2003).

Florel, which is an ethylene-releasing growth regulator, is the most promising of the growth regulator chemicals (Johnson 1989; Shamoun and DeWald 2002; Shamoun et al. 2003). The formulation to release ethylene is called ethephon (2-chloroethyl phosphoric acid). This growth regulator is registered by the Environmental Protection Agency and marketed as Florel® for use on conifers and fruit trees Hawksworth and Johnson 1989; (Hawksworth and Johnson 1998; Shamoun and DeWald 2002; Shamoun et al. 2003). Florel® induces shoots, flowers, and fruit abscission on dwarf mistletoe (Johnson 1989). There has been conflicting evidence on its effectiveness. Florel® may slow dwarf mistletoe spread and help protect the overstorey, but it does not kill the mistletoe plants (endophytic system of dwarf mistletoe) that have already infected trees (Hawksworth and Johnson 1989). Treatment with Florel® in stands infected with dwarf mistletoe resulted in resprouting of shoots from the endophytic system and long-term protection from dwarf mistletoe spread and intensification was not provided (Shamoun and DeWald 2002; Shamoun et al. 2003). Florel® can reduce dwarf mistletoe seed production for 2–4 years on light to moderately infected trees (Johnson 1989; Shamoun and DeWald 2002; Shamoun et al. 2003). To maintain longer control of dwarf mistletoe, re-spraying the ethephon would be necessary. Ethephon can be applied aerially by helicopter and by

ground sprays using a hydraulic sprayer, which can result in relatively high costs ($3–$4 per tree at 1989 costs) (Hawksworth and Fletcher 1989). This chemical is rarely used because of its high costs; it may be more suitable for high-valued trees where pruning is not possible (Johnson 1989; Hawksworth and Johnson 1989; Shamoun and DeWald 2002; Shamoun et al. 2003).

1.3.3 Genetic Resistance

In forest systems some trees within a species population will show few or no symptoms after being exposed to a disease; these trees are considered "genetically resistant" (Burdon 2001). Genetic resistance to insects and diseases in general is classified as "vertical" when specific genes operate to prevent initial establishment of the pathogen (Burdon 2001). Such resistant genes are often dominant. However, the parasite may acquire new virulence genes (which are typically recessive) either by mutation or immigration, and thus overcome the resistance attributable to major resistance genes (Burdon 2001). Alternatively, such virulence genes may be present at low frequency in the original parasite population and increase rapidly when an R-gene is introduced in the host population. Resistance in a host may also be attributable to genes that retard infection by reducing sporulation, extending the latent period, or reducing the rate of spread of the pathogen within host tissues. Such effects are typically conditioned by large sets of genes, and the resulting resistance is termed horizontal. Such genes tend to be less spectacular in their results giving rise to partial resistance. "Horizontal" genes tend to be less pathosystem specific (Burdon 2001). Consequently, the number of genes that contribute to genetic resistance in the host has a direct effect on the pathogen's ability to overcome resistance. Roth (1974a, 1974b) suggests that horizontal resistance to pathogens is most likely controlling resistance of western conifers to dwarf mistletoe.

There have been a number of field observations of mistletoe-free trees in areas heavily infected with dwarf mistletoe. Some examples of mistletoe-free trees include reports on Pinus ponderosa in areas heavily infected with *A. vaginatum* (Hawksworth 1961); *Pinus ponderosa* and *Pinus jeffreyi* (Scharpf and Parmeter [technical coordinators] 1978; Scharpf 1984, 1987); *Pseudotsuga menziesii* free of *A. douglasii* (Nowicki et al. 1999); and *Tsuga heterophylla* free of *A. tsugense* (Smith et al. 1993). In most cases, the progeny

of these resistant trees have not been tested for resistance. This would indicate a variation of resistance to dwarf mistletoe in the wild population (Smith et al. 1993; Shamoun and DeWald 2002; Shamoun et al. 2003).

Genetic improvements of tree species for dwarf mistletoe resistance through breeding programs or the selection of resistant scions have shown improvement in resistance in some species of trees; in other tree species there has been no change in dwarf mistletoe resistance (Shamoun and DeWald 2002). One test for resistance includes the collection of scions from resistant trees in the field. These scions collected are grafted to root stock and then tested for resistance. These grafted trees were out planted in a heavily infected mistletoe area. Studies with *Pinus ponderosa* and *Tsuga heterophylla* reported high correlations between resistant parents and their scions (Hawksworth and Edminster 1981; Scharpf and Roth 1992; Smith et al. 1993).

Examples of positive gains with progeny testing for dwarf mistletoe resistance include work with *Pinus ponderosa* seedlings. The seedlings from resistant parents have fewer infections and faster growth rates than seedlings from susceptible parents (Roth 1974a, 1974b). Work with the resistant progeny of *Pinus jeffreyi* trees and *Pseudotsuga menziesii* indicated that resistance was heritable (Scharpf and Roth 1992; Hawksworth and Edminster 1981; Nowicki et al. 1999).

Early studies suggest there may be significant variation in resistance to western hemlock dwarf mistletoe in conifer trees in British Columbia (Shamoun et al. 2003). Shamoun and Cartwright screened hemlock seedlings derived from seed orchards in Oregon, Washington, and British Columbia for provenance and family resistance to dwarf mistletoe. No trends in susceptibility to *A. tsugense* were detected to date (Cartwright et al. 2004). Resistant trials for *A. tsugense* are a relatively new program in the province. Other researchers are also interested in developing genetic breeding programs for resistance to dwarf mistletoe due to the change in harvesting systems in British Columbia and the Pacific Northwest (Shamoun and DeWald 2002; Shamoun et al. 2003).

Future work in genetic screening may include biotechnology approaches such as use of DNA markers for resistant trees. These markers can be used to select mistletoe-resistant trees that will eliminate the long generation times currently used to screen genetic resistance (Hadley 2001; Shamoun and DeWald 2002; Shamoun et al. 2003). In

British Columbia, the Ministry of Forests and Range and Pacific Forestry Centre have not yet certified resistant trees and therefore no DNA markers for resistant trees from selected resistance provenances have been determined (personal comm. B van der Kamp, 2006). Vertical genes in dwarf mistletoe may play some role in dwarf mistletoe resistance. Resistance studies to western spruce budworm defoliation of interior Douglas fir indicate partial genetic differences in different tree families (Chen et al. 2001). Perhaps a similar genetic variation occurs in dwarf mistletoe resistance.

1.4   Biological Control

The implementation of variable retention, along with management of riparian reserves in stands infected with dwarf mistletoe, can greatly increase the intensity of dwarf mistletoe, by opening the crown for increased light that can activate latent infections. Trees infected with dwarf mistletoe on variable retention borders can increase the spread of disease to newly generated adjacent stands (Muir 2002; Shamoun and DeWald 2002; Shamoun et al. 2003). Variable retention silvicultural systems can reduce control options for dwarf mistletoe compared with clear cutting silvicultural systems (Muir 2002; Muir et al. 2004). In variable retention, alternative methods of dwarf mistletoe control become important and researchers are investigating the possibility of using biological control agents in British Columbia (Shamoun and DeWald 2002; Shamoun et al. 2003).

Many diseases such as dwarf mistletoe are difficult and very expensive to control. In some cases, it can be impossible to control forest pests with methods that are traditional. Chemicals used for forest pests control can consist of non-selective compounds, which can damage non target plants and may leach out of sandy or gravelly soils, or consist of compounds that give top growth control only. In addition, because of leaching, chemicals should not used on weeds that grow close to bodies of water. Other concerns with the use of chemicals include contamination of soil and food, injury to endangered species and strong public criticism due to health concerns from such contaminations. In some cases, chemical controls are no longer available to the industry due to government banning of certain chemicals. Biological control of pests with their natural enemies can reduce our dependence upon toxic chemical chemicals and can prove to be an alternative method of forest pest and disease control (Charudattan 1989, Templeton 1989, Green et al 1998).

Biological weed control is the deliberate use of natural enemies to suppress the growth or reduce the population of weed species (Watson 1998). Biological control of weeds can involve two approaches 1) classical (inoculative) biological control and 2) inundative biological control (bioherbicide). A bioherbicide is a living inoculum of a plant pathogen, formulated and applied to control or suppress the growth of the weed species (Watson 1998).

The use of the classical approach is directed mainly toward the control of exotic pests, which have spread into area in the absence of natural enemies. Control is achieved by the importation and release of host-specific pathogens virulent to the target pest in its native region (Green 2003; Templeton et al. 1979; Watson 1991). An example of successful classical biological control of a forest pest was the introduction *Septoria passiflorae* Syd. for the biological control of the exotic weed, banana poka (*Passiflora tripartitia* (Juss.) Poir var. *tripartita* Holm-Nie. Jorg. & LAW), at different forest sites in Hawaii with over 50 percent biomass reduction of the weed 3 years after inoculations (Trujillo et al. 2001).

Inundative biological control relies on the use of natural enemies to control pests. The use of the inundative approach is based on the principles of epidemiology which includes the pathogen, environment, or plant factors often affect plant disease (Watson 1998). The inundative approach tries to enhance disease development by supplying abundant inoculum onto a susceptible host (Charudattan 1988; Watson 1998). The application is timed to take advantage of favourable environmental conditions at the most susceptible growth stage of the pest (Charudattan 1988, 1990).

An example of successful inundative biological control agent is *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. f. sp. *malvae*, under the trade name BioMal^{TM}, used to control the weed round-leaved mallow (*Malva pusilla* Sm.) in strawberry field crops (Table 1.3) (Makowski et al. 1989; Mortensen 1989; Mortensen and Makowski 1990).

The development of potential biological control agents begins with the discovery of a diseased plant pest found in the field or in the greenhouse (TeBeest et al. 1992; TeBeest and Templeton 1995; Watson 1998). Fungal causal agents are cultured on artificial media, identified and re-inoculated back on the target organism. Potential candidates are further tested for infectivity, pathogenicity and small scale test (Charudattan 1988, 1990).

1.4.1 What Makes a Good Biological Control Candidate?

The most important consideration in selecting a potential biological control agent for a plant disease is the efficacy of the potential biological control. Efficacy should be assessed by the speed, amount, and ease of disease control (Charudattan 1990).

Factors such as ease of dissemination or dispersal, the infection process, and survival of the pathogen are also important for potential biological control agents. Preferred characteristics of a potential biological control agent include (1) vigorous growth and sporulation on artificial media, (2) virulent to target, (3) genetically stable, (4) restricted host range, (5) tolerance to a broad range of environmental conditions, (6) prolific propagules production, and (7) innocuous in ecological effects (Templeton et al. 1986; Charudattan 1990).

Biological control agents will not eradicate the pest; however, they will reduce its vigour, abundance, and spread (Charudattan 1990; Shamoun and DeWald 2002; Shamoun et al. 2003). The goal of applying biological control to an infested crop or forest is to control the pest to a level that will reduce or eliminate the economic damage. If biological control is successful, pests become increasingly rare and the biological control agent population also declines until a new equilibrium forms between the agent and pest (Templeton et al. 1986).

Table 1.1 and 1.3 are examples of biological control agents for the management of agricultural weeds and forest competing vegetation has seen some success. In Canadian forests, native isolates of *Chondrostereum purpureum* (Pers: Fr) are used to control unwanted woody shrubs. *C. purpureum* is also used in Europe as BioChon[TM], in South Africa as Stumpout[TM] (Morris et al. 1999), and in eastern Canada as Myco-tech[TM]. These formulations were developed in a collaborative research project between MycoLogic Innovative Biological controls and Dr. S.F. Shamoun of the Canadian Forest Service. Recently *C. purpureum* strain PFC 2139 has been registered in the United States as Chontrol[TM] (collaborative research project between MycoLogic Innovative). This registered product was used in conifer reforestation sites and utility rights of ways to control weedy hardwoods (Wall 1994; Shamoun et al. 1998; Becker et al. 1999; Harper et al. 1999; Shamoun and DeWald 2002). Epidemiological evidence shows that the application of this product did not threaten susceptible crops cultivated >500 m from the

treatment area (Shamoun 1998; Shamoun and DeWald 2002; Shamoun et al. 2003; Green 2003).

Table 1-1 Examples of forestry biological control agents, target disease and authors and produces.

Target fungus	Disease	Biocontrol fungus	Authors/Producers
Chondrostereum purpureum	*C. purpureum* and other soil and foliar pathogens	*Trichoderma viride* Pers ex. Gray	Trichodowels, Trichoject, Trichoseal, and others, Agrimms Biologicals New Zealand and others (Butt et al. 2001)
Armillaria mellea (Vahl ex Fr.) Kumm.	Root disease: trees	Trichoderma viride	Raziq and Fox 2003
Armillaria ostoyae	Root disease: trees	*Hypholoma fasciculare*	Chapman and Xiao 2000
Heterobasidion annosum	Root disease: trees	*Trichoderma viride*	Binab T, Bioinovation Sweden (Butt et al. 2001)
Heterobasidion annosum	Root disease: trees	*Phlebiopsis gigantea* (old name *Peniophora gigantea*)	Rotspot, Kermira, Agro Oy, Finland (Butt et al. 2001)
Pythium spp. J. Schröt.	Damping off: seedlings	*Trichoderma harzianum* Rifai	T 22, and T22HB, Biotrek, Root ShieldBioworks = (TGT) Genena, USA (Butt et al. 2001)
Phytophthora cinnamomi Ronds	Root rot: trees and herbs	*Leucopaxillus* spp. (Cost. & Duf.)	Marx 1969
Cronartium ribicola J.C. Fisch.	Blister rust: pines	*Tuberculina maxima* Rostri.	Kimmey 1969
Cronartium spp.	Blister rust pines	*Sphaerellopsis filum* (Biv.) B. Sutton	Yuan et al. 1999, Miyagawa and Unoue 1996
Acacia spp., weed species control	Weedy exotic in South Africa	*Chondrostereum purpureum*	Stumpout® Morris et al. 1999
Weedy hardwood spp. competing vegetation in conifer sites and right of way	Disease of hardwoods in North America	*Chondrostereum purpureum*	MYCO-TECH™ and Chontrol™ and PFC 2139 Shamoun 2003; EPA Pesticides-Fact sheet
Ophiostoma novo-ulmi (Buisman) Nannf	Chestnut blight	*Ophiostoma ulmi* (hypovirulent fungi)	Bernier et al.1996
Gremmeniella abietina (Lagerb.) M.	Pine shoot blight	*Phaeothecea dimorphospora*	Yang et al. 1993; 1995

The biological control agent *Phlebiopsis gigantea* (Fr.:Fr.) is used to control the forest disease *Heterobasidion annosum* (Fr.) Bref. *H. annosum* causes root and butt disease and is found throughout northern temperate hemisphere, on over 200 woody species (Barnard 1999; Roy et al. 2003). *H. annosum* is a fungal disease that is spread largely through infestations of freshly cut stumps and into the roots stumps and natural root contacts allowing the fungus to spread to larger area (Barnard 1999; Roy et al. 2003; Berglund 2005). Research on the biocontrol agent *P. gigantea* by Roy et al. (2003) used freshly cut red pine stumps and inoculated the stumps with *H. annosum* and *P. gigantea*.

The biological control agent *P. gigantea* inhibits the colonization by *H. annosum*. *P. gigantea* is registered as Rotstop^{TM} in Finland, Sweden, Norway, Switzerland, and the United Kingdom for commercial use (Roy et al. 2003; Berglund 2005).

The biological control agent *Hypholoma fasciculare* (Huds. Ex Fr.) has been studied for the control of *Armillaria ostoyae* (Romagn.) Henk., which is a serious root disease in southern British Columbia, Canada, and around the world (Allen et al. 1996). Field trials with heavy *Armillaria ostoyae* infections were inoculated with bagged inoculum of *H. fasciculare* in a mixture of sawdust and bran. The inoculum bag was buried adjacent to the base of the bole and a large root stump. *H. fasciculare* invaded the fresh stumps even with *A. ostoyae* present. After 2 years, *H. fasciculare* was colonizing roots 1.6 m from the bole of the treated roots. Results from this trial appear promising but it is too early to determine the effects of this biological control agent (Chapman and Xiao 2000).

1.4.2 Constraints in the Development of Bioherbicides

To date there are approximately less than twenty five bioherbicides that are commercially available in North American markets despite the need for bioherbicides and the considerable amount of research with bioherbicides. There are number of constraints that have hampered the development of bioherbicides. Bioherbicides need to be fast-acting, predictable, environmentally safe, and provide similar weed control as comparable chemical herbicides (Mortensen 1998). The most important factors to consider are the risks involved in releasing bioherbicides into the environment. Their safety, persistence in the environment, potential genetic changes are major concerns of scientists and regulatory bodies. The four major constraints that are slowing the progress in developing

bioherbicides include 1) biological 2) environmental 3) technological and 4) commercial limitations.

1.4.2.1 Biological constraints

Bioherbicides are less commonly used compared to herbicides since most have a narrow weed target range. Many bioherbicides are host specific. This can be an advantage where a weed is closely related to the crop in which it is to be controlled. In other situations this may be a disadvantage where there are a number of different weeds present in a crop situation. To extend or increase the host range you can combine biological agents, addition of plant extracts to the formulation, or addition of chemical herbicides that are compatible with the biological control, and wound plant surfaces to slightly increase the efficacy of the bioherbicide. It may also be possible to manipulate the genes to genetically modify the bioherbicide (Mortensen 1998; Watson 1998).

Host specificity can be a restraint when the biological control agents are not host specific. For the bioherbicide, Collego™ several non target species of *Aeschynomene, Pisum sativum, Lupinus densiflorus, Vicia faba* and *Lathyrus* spp have been found to be susceptible to *C. gloeosporioides f. sp. aeschynomene*. Labeling restrictions are required for such bioherbicides to prevent damage such as over spray and wind dispersal to non target species (Charudattan 1988).

Genetic make up of the target weed and the pathogen are often over looked. The genes of disease causing pathogens are usually highly specific. The plant pathogen genes provide a means for a pathogen to overcome the defense system of one particular type of plant. The invasion genes of the pathogen have to match the defense genes of the weed. This plant-pathogen interaction results in a specific weed attack (Charudattan 1990).

Genetic factors such as the population diversity of the target weed are important prior to the production and release of the biological control agent. This is necessary to determine whether a single isolate can be applied across a large area or if treatment must be regionalized using local isolates collected from each region. Ideally, pathogen isolates chosen for development should be genetically similar to local populations of the plant pathogen to reduce the risk of introducing novel alleles into the local ecosystem (Hintz et. al. 1996).

The biology and morphology of the target weed can influence the level of control achieved (Mortensen 1998). For instance, grass weeds are harder to control than Dicot weeds because of the growing points are well protected from infection of the foliar pathogens. Understanding the biology and the weakest links in the target weed are important. For example with the *Taraxacum officinale* (dandelion), four week old seedlings were the most susceptible to the pathogen *Phoma herbarum* (Neumann et. al. 1999). In other cases, bioherbicides are more effective or enhanced when the weed is in competition with the crop (Mortensen 1998).

Scientists have debated the potential for build up of weed resistance to bioherbicides, but so far, this is not a concern. Devine and Collego have been in use for at least two decades with no appearance of resistant or immune weed genotypes (Charudattan 1990).

1.4.2.2 Environmental constraints

The two most important environmental factors that dictate the application of bioherbicides include moisture and temperature. Timing of bioherbicide can be restricted due to limited humidity, dew period, and moisture conditions of the site as well as the ambient temperature. Formulations that have water-retaining materials can reduce time restrictions for infection and make the bioherbicide less dependent on environmental conditions such as water for initial infection (Mortensen 1998).

Other environmental consideration that must be considered when applying a bioherbicide is compatibility with other herbicides in an integrated pest management system. The interaction of bioherbicide agents with microorganisms inhabiting phyllosphere and leachates from plants can act antagonistic to the herbicide (Mortensen 1998).

There are some concerns when introducing a large number of the bioherbicides propagules into the environment. In most cases the population dynamics of the microorganism in the soil or plant environment are unknown. It is also unknown how these microorganisms interact with other species and biological systems in the environment. There is the possibility that these microbes could displace non-target microorganisms (Hintz et. al. 1996; Mortensen 1998). There has been a lack of monitoring of the environment where these bioherbicides have been applied. It is unknown what effects they may be causing in displacing native species in the

surrounding ecosystem. There is also limited information on the effect gene transfer (Lynch 1992).

Applying bioherbicides may pose some risks to human health. These may include allergic reactions to fungal pathogens or to the formulation products such as wheat or other adjuvants (Lynch 1992).

1.4.2.3 Technological constraints

Mass production of viable, infective and genetically stable propagules of a plant pathogen is essential in the development of a bioherbicide (Mortensen 1998). To effectively mass-produce a bioherbicide the propagules should be adapted to submerged fermentation production since it is the most cost effective. This means that alternative methods of mass production should be developed for fungi that do not produce well in liquid media.

The science involved in formulating the bioherbicide is one the most challenging. Research is required in development of more reliable and efficacious bioherbicides. Efficacy encompasses the level of weed control as well as the speed and ease in which weed control is accomplished. Unless the bioherbicide is fairly fast acting, and achieves similar levels of control as chemical herbicides and is easy to use with predictable performance in the field, it is difficult to expect acceptance by industries and users (Charudattan 1988).

This may entail combining the bioherbicide with chemical herbicides or growth promoters. Another challenge is to keep the propagules viable and infective for one to two year periods. This includes research in physiology of spore dehydration and dehydration (Bannon et al. 1990). The delivery of the bioherbicides should also be compatible with existing farm equipment. Adjuvants added to the bioherbicides should improve adhesion and distribution of the propagules on the plant surface; enhance spore germination, germ tube formation, and appressorium formation (Boyetchko 1998; Mortensen 1998).

1.4.2.4 Commercial limitations

The market for bioherbicides can be limited because it usually targets one weed. Small markets have deterred many industries from getting involved due to the limited

opportunities to recover the cost of producing a bioherbicide (Mortensen 1998). The costs of development for a bioherbicides is approximately $1.5- 3.0 million dollars a year. The costs to develop a chemical herbicide cost, $ 30 million dollars making bioherbicides cheaper and favorable to develop (Charudattan 1988 Watson 1998 and Mortensen 1998).

Patent protection costs is an important requirement for potential bioherbicides to guarantee the recovery of development and registration costs by a company willing to invest the time, effort and money in making the new technology available to the public (Mortensen 1998). The cost of patenting limits the exchange of ideas and results between competing scientists and is controlled until the patent takes effect. This restricts communication and delays the publishing of research work that could result in slowing the advancement of this science and technology (Mortensen 1998).

Since bioherbicides are treated as chemical herbicides they are regulated in Canada under the Pest Control Act and Regulations. The agency responsible for registration of microbial pest agents in Canada is called the Pest Management Regulatory Agency (PMRA). In the United States, EPA regulates the registration of pesticides including pest control. Increases in the restriction and costs of registration have discouraged the private sector from investing in the development of bioherbicides.

1.4.3 Hyperparasites of Dwarf Mistletoe (*A. tsugense*)

Many fungal hyperparasites are associated with dwarf mistletoes. Fungi that attack flowers, shoots, and fruits of dwarf mistletoes include *Cylindrocarpon gillii, Caliciopsis areuthobii,* and *C. gloeosporioides. Neonectria neomacrospora* and *Cytospora abietis* are canker fungi associated with endophytic system of the dwarf mistletoe (Hawksworth and Geils 1996; Shamoun and DeWald 2002; Shamoun et al. 2003).

Cylindrocarpon gillii (Ellis) J.A. Muir (formerly *Septogloeum gillii*) is a fungal parasite that can attack both shoots and flowers of dwarf mistletoe. *Cylindrocarpon gillii* can be found on *Arceuthobium abietinum* Engelm. ex Munz f. sp. *concoloris* Hawksw.& Wiens, *A. abietinum* Engelm. ex Munz f. sp. *magnificae* Hawks. & Wiens, *A. americanum, A. apachecum* Hawks. & Wiens, *A. blumeri* A. Nels., *A. californicum* Hawks. & Wiens, *A. campylopodum* Engelm. in Gray, *A. cyanocarpum* (A. Nels ex Rydb) A. Nels, *A. divaricatum* Engelm (Pinyon dwarf mistletoe), *A. douglasii, A. laricis, A.*

microcarpum (Engelm) Wiens & Nickrent, and *A. tsugense* (Muir 1973; Shamoun 1998; Kope 1997; Kope and Shamoun 2000). Early symptoms are small, yellowish-white lesions on shoots, which gradually coalesce and erupt through the epidermis, releasing a mass of white spores (Hawksworth et al. 1977; Shamoun and DeWald 2002). The mode of action of *C. gillii* is to invade the shoot tissue of dwarf mistletoe with the exception of the vascular elements. The shoots are girdled, killing the distal portion of the shoot (Shamoun and DeWald 2002). Previous studies Hawksworth et al. 1977; showed that *C. gillii* is not a wound parasite and it is able to penetrate unwounded tissue (Shamoun and DeWald 2002; Shamoun et al. 2003). This fungus also produces an abundance of inoculum in culture. Mielke (1959) inoculated *A. americanum* with *C. gillii* and found that the parasite vanished after 3 years. The lack of rain and cool weather suggested that the climate conditions were not conducive for *C. gillii* (Hawksworth et al. 1977; Shamoun and DeWald 2002).

Caliciopsis arceuthobii [Peck] ME Barr (Barr et al. 1986), (formerly W*allrothiella arceuthobii* [Pk] Sacc.) is a hyperparasite to the pistillated flowers of *Arceuthobium pusillum Peck*, and *A. douglassi* in the United States and Canada as well as *A. americanum* and rarely, *Arceuthobium vaginatum* (Willd.) Presl ssp. *cryptopodum* (Englem.) Hawks. & Wiens (Dowding 1931; Kuijt 1969b; Knutson and Hutchins 1979 Shamoun and DeWald 2002; Shamoun et al. 2003). In the spring the main source of inoculum for this hyperparasite are the ascospores, which are carried by insects, wind, or rain to infect the flowers of dwarf mistletoe (Wicker and Shaw 1968). After 2 months, hyphae penetrate the fruits to the ovary wall and normal fruit and seed dispersal are reduced. *C. arceuthobii* is distributed throughout Western Canada, United States, and Mexico (Kuijt 1963; Hawksworth et al. 1977; Shamoun and DeWald 2002). Potential for biological control is limited, since annual fluctuations in natural infections are large and it is difficult to produce *in vitro* (Wicker and Shaw 1968; Hawksworth et al. 1977; Shamoun and DeWald 2002; Shamoun et al. 2003).

Other fungi parasitic to dwarf mistletoe shoots that could be biological control agents include *Alternaria alternata* (Fr.) Keissl., *Aureobasidium pullulans* (de Bary) G. Arnaud, *Coniothyrium* sp., *Metasphaeria wheeleri* Linder, *Pestalotia maculiformans, Pestalotia maculiformans* Guba & Zeller, *Pestalotia heterocornis* Guba, and *Phoma* sp.

(Hawksworth et al. 1977; Hawksworth and Wiens 1996; Shamoun 1998; Kope and Shamoun 2000). Little to no research has been done to determine if these fungi are successful candidates for the control of dwarf mistletoe (Shamoun and DeWald 2002; Shamoun 2003).

Cytospora abietis Sacc. is a canker fungus associated with dwarf mistletoe swelling on *Abies magnificae* A. Murr. and *Abies concolor* (Gord & Glend.) Lindl. ex Hildebr. parasitizing *A. abietinum* (Wright 1942; Hawksworth 1972; Shamoun and DeWald 2002; Shamoun 2003; Shamoun et al. 2003). This canker-causing fungus can invade the dwarf mistletoe swelling and kill the host branch. On occasion, *C. abietis* will parasitize non-infected host branches and has the potential to harm the host tree. Further research will be required before *C. abietis* can be developed into a biological control. The efficacy of this biological control agent is unknown (Hawksworth 1972; Shamoun and DeWald 2002; Shamoun et al. 2003).

Neonectria neomacrospora (Booth and Samuels) Mantiri & Samuels (formerly *Nectria neomacrospora* Booth & Samuels) is another canker fungus that parasitizes dwarf mistletoe swellings (Funk 1973; Funk 1981; Shamoun and DeWald 2002; Shamoun et al. 2003; Rietman 2004). This hyperparasite has been observed on *Arceuthobium occidentale* Engelm. on pine, *A. abietinum* on white fir, and *A. tsugense* swellings on hemlock and shore pine (Shamoun and DeWald 2002; Shamoun et al. 2003). *N. neomacrospora* has an anamorph stage, which includes conidia borne in a sporodochia, and a teleomorph stage, which includes a stroma with dark red perithecia (Shamoun and DeWald 2002). This fungus is thought to infect the endophytic system of the dwarf mistletoe (Shamoun and DeWald 2002; Shamoun et al. 2003; Rietman 2004).

Typical symptoms and signs associated with *N. neomacrospora* include resinosis, sunken cankers, and in some cases, girdling and death of the infected branches (Shamoun and DeWald 2002; Shamoun et al. 2003; Rietman 2004). Other symptoms of this fungus include a significant reduction in healthy shoots, and slow growth and elongation of mistletoe swelling. *N. neomacrospora* may require 1–2 years to establish an infection within the bark of the dwarf mistletoe infected hemlock (Shamoun and DeWald 2002; Shamoun et al. 2003; Rietman 2004). Further research is required to determine if *N. neomacrospora* can infect dwarf mistletoe swelling and kill the endophytic system. Death

of the endophytic system would result in long-term control of dwarf mistletoe (Rietman 2004; B. van der Kamp, pers. comm., 2005).

1.5 Western hemlock (*Tsuga heterophylla*)

T. heterophylla is the dominant forest tree within its range. In low elevations coastal forests of Washington, Oregon, and southern British Columbia, it shares dominance with *Picea sitchensis* (Sitka spruce) (Meidinger and Pojar 1991; B.C. Ministry of Forests. 1999; Packee 2002). On slightly drier sites of the western Cascades and Vancouver Island its primary codominant is *Pseudotsuga menziesii* (Douglas-fir) (Pojar and MacKinnon 1994; B.C. Ministry of Forests. 1999; Packee 2002). Other species of trees commonly found growing with western hemlock within the Coastal Western Hemlock biogeoclimatic zone (CWH) are western red cedar (*Thuja plicata* Donn ex D. Don), yellow-cedar (*Chamaecyparis nootkatensis* (Don) Spach) at higher elevations, and various deciduous species (Meidinger and Pojar 1991; B.C. Ministry of Forests. 1999; Packee 2002). These trees create multi-layered, multi-aged forests where large living and dead trees create many habitats vital to support organisms such as birds, small animals, and fungi (Tinnin et al. 1982; Tinnin and Forbes 1999; Geils and Hawksworth 2002).

1.5.1 Western Hemlock (*Tsuga heterophylla*) Ecology

Western hemlock (*Tsuga heterophylla* [Raf.] Sarg.) thrives in the humid to dry areas of the Pacific coast and northern Rocky Mountains. Western hemlock is an important commercial species for British Columbia, making up 40% of the total timber harvested in the coastal regions of the province (Webber 2000). Home building components such as doors and other structural components are milled from western hemlock timber due to the wood's even grain. Pulp and paper are also made from western hemlock (Webber 2000; Packee 2002).

Western hemlock is found on wet to dry sites and is well adapted to grow on humus and decaying wood. It also grows on mineral soil, raw floodplains, or recently deglaciated sites (Meidenger and Pojar 1991; Packee 2002). Western hemlock is shade tolerant and is found on low to mid elevations from 0 to 1800 m. It ranges along the Pacific coast from southeastern Alaska to central California. Inland, it grows along the western and upper eastern slopes of the Cascade Range in Oregon, Washington, and the

west side of the Continental Divide of the northern Rocky Mountains in Montana and Idaho north to Prince George, B.C. (Meidenger and Pojar 1991; B.C. Ministry of Forests. 1999; Packee 2002).

In British Columbia, western hemlock is found in the Coastal Douglas-Fir (CDF), Coastal Western Hemlock (CWH), Mountain Hemlock (MH), and Interior Cedar–Hemlock (ICH) biogeoclimatic zones (Meidinger and Pojar 1991). Hemlock dwarf mistletoe is found throughout the CDF and CWH zones where western hemlock stands are found. It has not been detected in the ICH zone (Baranyay and Smith 1974; Smith 1977).

The CWH biogeoclimatic zone occurs at low to middle elevation mostly west of the coastal mountains, along the entire B.C. coast and on to both Alaska and Washington/Oregon. The zone covers much of Vancouver Island, the Queen Charlotte Islands, and the Coast Mountains (Meidenger and Pojar 1991; Pojar and MacKinnon 1994). It also penetrates the coastal mountain barrier somewhat in major river valleys, especially along the Fraser and Skeena rivers (Meidenger and Pojar 1991). The CWH occupies elevations from sea level to 1830 m on windward slopes in the south and mid-coast and 300 m in the north (Pojar and MacKinnon 1994). The CHW zone has one of Canada's wettest climates (mean annual precipitation: 1000–4400 mm) and most productive forests (Meidenger and Pojar 1991; Pojar and MacKinnon 1994; Packee 2002).

The common stand disturbances in western hemlock forests are wind throw and various diseases (Packee 2002). Western hemlock seedlings are adapted to organic substrates, especially downed wood, which gives the hemlock a significant competitive advantage over other conifers in the area (Meidinger and Pojar 1991). Western hemlock is generally known as a climax species either alone or in combination with other shade-tolerant tree species (Meidinger and Pojar 1991; Kimmins and Pojar 1994; B.C. Ministry of Forests. 1999; Packee 2002).

In full light western hemlock can grow quickly, can survive in a variety of seedbeds, and is considered a pioneer plant (Packee 2002). It also invades several stages of forest succession after a forest canopy has formed. A pure hemlock stand can result from a mixed stand where hemlock is commonly found after several centuries without a major

disturbance. The growth of western hemlock can remain suppressed under an overstorey for up to 60 years (Pojar and MacKinnon 1994; Packee 2002). Removal of the overstorey results in vigorous young hemlock stands, especially under the height of 1.4 m (Packee 2000). On drier slopes, western hemlock achieves dominance but rarely replaces western red cedar entirely (Meidinger and Pojar 1991; Packee 2002).

The CWH zone probably encompasses the greatest diversity and abundance of wildlife habitat of any ecological zone in British Columbia (Meidinger and Pojar 1991; Douglas 2003). Many species rely on the habitat that the old forest provides in this ecological zone (Douglas 2003). Within the CWH, riparian ecosystems are found on the margins of rivers, streams, lakes, and marshes, as well as the floodplains of large rivers, small streams, and gullies (Douglas 2003). Riparian ecosystems are closely linked to habitat for fish and other aquatic organisms (Kimmins 1997). Many aquatic and forest organisms rely on riparian ecosystems for their survival. Five species of pacific salmon depend on areas for spawning and rearing within the CWH. Damage to fish habitat and water quality may increase with the removal of forests in these riparian zones (Douglas 2003).

Western hemlock in the CDF zone is found on very dry soils, in very poor to medium soil nutrient regime, and in some cases boggy sites. On most western hemlock sites the tree canopy is interrupted due to the rock outcrops or shallow soils (Wass 1976). On the western hemlock sites where trees are well developed, Douglas-fir will often regenerate (Wass 1976; Packee 2002). Many sites in the CDF zone on Vancouver Island are not dedicated to forestry; however, large parcels of private lands have been managed for timber (Nuszdorfer et al. 1991).

1.5.2 Western Hemlock Dwarf Mistletoe (*Arceuthobium tsugense* ssp. *tsugense*)

Western hemlock dwarf mistletoe, *Arceuthobium tsugense* (Rosendahl) G.N. Jones ssp. *tsugense*, is an important parasite of a number of economically important conifers in the western United States and Canada (Unger 1992). This mistletoe will infect 12 host species under natural conditions and 20 species using artificial inoculations (Nickrent and Butler 1990). The effects of dwarf mistletoe on its host are reduced growth rates, vigour, and increased susceptibility to tree diseases and reduced wood quality. In moderately to

severely infected stands, where greater than 50% of the branches are infected, stem growth is estimated to be reduced by 20–40% (Thomson et al. 1985; Muir 2003).

The impact of western hemlock dwarf mistletoe is difficult to assess on coastal regions of British Columbia. In Washington and Oregon, inventory of western hemlock dwarf mistletoe is estimated to reduce the growth of hemlock forests by 4.5% annually (Muir et al. 2004). This implies that in the B.C.'s CWH biogeoclimatic zone, which consists of 8 million ha of predominately hemlock forests with a mean increment of 6 m³/ha/yr, western hemlock dwarf mistletoe could cause an estimated growth loss of 2 million m³/yr (Muir 2003).

1.5.3 Hosts of Hemlock Dwarf Mistletoe (*A. tsugense ssp. tsugense*)

1.5.3.1 Subspecies of hemlock dwarf mistletoe (*A. tsugense ssp. tsugense*)

There are two subspecies of *A. tsugense*: western hemlock subspecies (*A. tsugense ssp. tsugense*) and mountain hemlock dwarf mistletoe subspecies (*A. tsugense* ssp. *mertensianae*). Figure 1.5 is photograph of a healthy *A. tsugense* spp. *tsugense* swelling. Table 1.2 summarizes the hosts, distribution and shoot height of the subspecies of western hemlock dwarf mistletoe. Each has different host tree preferences and differs in shoot height (Hawksworth and Weins 1996; Mathiasen and Daughterty 2005). Due to the extreme reduction in morphological characteristics with parasitic plants molecular systematics can be useful in determining the species, subspecies, and genus relationships in *Arceuthobium*. The isoenzymes data strongly supports the concept of two biological separate subspecies for *A. tsugense* ssp. *mertensianae* and *A. tsugense* spp. *tsugense* (Nickrent and Butler 1990; Hawksworth and Wiens 1996).

Table 1-2 Hosts, distribution and shoot height of subspecies of western hemlock dwarf mistletoe (*A. tsugense*) (Hawksworth and Wiens 1996; Wass and Mathiasen 2003; Mathiasen and Daughterty 2005).

	Western hemlock dwarf mistletoe	Mountain hemlock dwarf mistletoe
Common name	Western hemlock dwarf mistletoe	Mountain hemlock dwarf mistletoe
Scientific name	*A. tsugense* ssp. *tsugense*	*A. tsugense* ssp. *mertensianae*
Primary hosts	Western hemlock (*Tsuga heterophylla,* Shore pine (*Pinus contorta* Dougl ex Loud var. *contorta*)	Mountain hemlock (*Tsuga tsugense* ssp. mertensianae), Pacific silver fir (*Abies amabilis)*, Subalpine fir (*Abies lasiocarpa* (Hook) Nutt. (Merriam) Lemmon*)*, Noble fir (*Abies procera*)
Second-ary hosts		White bark pine (*Pinus albicaulis* Engelm.)
Occasional hosts	Noble fir (*Abies procera* (Hook) Nutt), Pacific silver fir, (*Abies amabilis* (Dougl.) Forbes), Mountain hemlock (*Tsuga tsugense* ssp. mertensianae)	Western white pine (*Pinus monticola* Dougl. ex Loud.)
Rare hosts	Englemann spruce (*Picea* enge*lmannii* Parry ex. Engelm., Sitka spruce (*Picea sitchensis* (Bong.) Carr), Douglas fir (*Pseudotsuga menziesii*) ,Western larch (*Larix occidentalis*), Western white pine (*Pinus monticola*), Grand fir (*Abies grandis* (Dougl.) Forbes), Subalpine fir (*Abies lasiocarpa*), Western white pine (*Pinus monticola)*	Brewer's spruce (*Picea breweriana* S. Wats), Lodgepole pine (*Pinus contorta* var. *latifolia*), Western hemlock (*Tsuga heterophylla*)
Immune	*Abies magnificae*, Western red cedar (*Thuja plicata*), *Pinus contorta* var. *murrayana* (Grev. & Balf.) Critchfield	Western red cedar (*Thuja plicata)*
Pheno-logy	Peak anthesis in August. Fruits mature from September to December.	Peak anthesis from mid-August to mid-September. Fruit matures in mid August to September.
Distribu-tion	Distributed from Haines, Alaska, to coast of B.C., Washington, Oregon, and northern California	Western Canada (southern B.C.) and western Washington. The distribution extends from Vancouver, B.C., through the Cascade Mountains of Washington and Oregon to central Sierra Nevada in Alpine County, California.
Shoot height	5–13 cm (mean 7 cm)	3–9 cm (mean 5 cm)

Figure 1-5 Healthy western dwarf mistletoe (*A. tsugense* ssp. *tsugense*) shoot with berries from the PFC field trial.

1.5.3.2 Subspecies of *A. tsugense* ssp. *tsugense*

The subspecies *A. tsugense* ssp. *tsugense* consist of two races on western hemlock and shore pine, respectively (Hawksworth and Wien 1996: Hawksworth et al. 2002). For the western hemlock race the principal host is western hemlock and shore pine is a rare host. The same trend occurs in the shore pine race where shore pine is the principle host and western hemlock is a rare host (Hawksworth and Wiens 1996; Hawksworth et al. 2002). Wass and Mathiasen (2003) found morphological and distribution differences between the two races. The western hemlock dwarf mistletoe race is distributed from southeastern Alaska throughout the coastal forests of British Columbia, Washington, Oregon, and northwest California (Hawksworth and Wiens 1996; Geils and Hawksworth 2002). This form occurs from sea level to 1200 m in elevation (Hawksworth and Wiens 1996; Wass and Mathiasen 2003).

The shore pine dwarf mistletoe race is found in southeastern British Columbia mainly along the eastern shore of Vancouver Island, along adjacent areas on the mainland coast, and on Orcas Island in Washington State. The western hemlock race has a wider distribution than the shore pine race (Nickrent and Butler 1990; Wass and Mathiasen 2003).

Wass and Mathiasen (2003) found that there are enough differences (both morphological, distribution of the races and phenology) between the two western hemlock races to warrant placing each race into two different subspecies. For example the flowering of the shore pine dwarf mistletoe occurs around the end of July and finishes around the first week of August while hemlock dwarf mistletoe flowers near the beginning of July and ends in the second week in August (Smith 1985; Wass and Mathiasen 2004; Wass and Mathiasen 2004). In general, shore pine dwarf mistletoe has a shorter flowering period than hemlock dwarf mistletoe. Seed dispersal for hemlock dwarf mistletoe starts at the end of August and can last up to the first week in November. For shore pine, the seed dispersal starts 2 weeks before the hemlock dwarf mistletoe and finishes in mid October (Wass and Mathiasen 2003; Wass and Mathiasen 2004). At present the differentiation of races or subspecies remains unresolved among dwarf mistletoe researchers with western hemlock dwarf mistletoe. Molecular systematics may determine the difference between the two races (Nickrent and Butler 1990).

1.5.4 Biology of Western Hemlock Dwarf Mistletoe (*A. tsugense spp. tsugense*)

Climatic events can limit seed production of western hemlock dwarf mistletoe. In areas where frost occurs, dwarf mistletoe shoots exhibit reduced seed production. Climatic conditions such as windstorms, snow, humidity, and ice can reduce seed production (Richardson and van der Kamp 1972; van der Kamp 1998). At higher elevations and latitudes there is a trend to reduce the intensification of western hemlock dwarf mistletoe due to the cooler weather (Richardson and van der Kamp 1972; Trummer et al. 1998; van der Kamp 1998). Spread and intensification of hemlock dwarf are slower in Alaska compared with the Pacific Northwest (Trummer et al. 1998). In Alaska, hemlock dwarf mistletoe spreads slowly, even with an infected overstorey and residual trees located adjacent to young growth trees (Trummer et al. 1998).

Shaw and Weiss (2000) note that the dwarf mistletoe physiological factors may vary with the height of the stands and individual tree crowns, and that these can affect several aspects of the life cycle of hemlock dwarf mistletoe including seed germination,

endophytic development, aerial shoot production, flowering, pollination, and seed production.

Complexes of biotic and abiotic factors are associated with the vertical structure of the forest stand that interacts with the production of dwarf mistletoe aerial shoots in the forest canopy (Shaw and Weiss 2000). Direct light to the hemlock dwarf mistletoe infection is an essential factor that can increase production of aerial shoots and seeds (Shaw and Weiss 2000). The dense foliage of western hemlock trees results in differences in light quality at different heights in the tree canopy (Smith 1977; B.C. Ministry of Forests 1983; Shaw and Weiss 2000; Wass 2006). Shaw and Weiss (2000) found that the highest PAR transmission was in the middle or upper canopy (15–30 m in older trees). Upper canopies of hemlock trees reduce the light to hemlock dwarf mistletoe on the lower branches, reducing the production of aerial shoots and seeds and spread of hemlock dwarf mistletoe (Shaw and Weiss 2000).

On good sites where tree height growth exceeds the vertical spread of the hemlock dwarf mistletoe, the upper tree canopies remain uninfected and shades the infections on the lower branches. On these sites the DMR rarely exceeds 2 to 3 and damage is minimal (Richardson and van der Kamp 1972; van der Kamp 1998; Muir 2002).

On poor or low sites hemlock trees grow at a slower rate allowing the upper canopy to be infected. On these sites, vertical and horizontal hemlock dwarf mistletoe spread can be rapid (Richardson and van der Kamp 1972; van der Kamp 1998; Muir 2002).

1.5.5 Life Cycle of Western Hemlock Dwarf Mistletoe (*A. tsugense* spp. *tsugense*)

The life cycle of *A. tsugense* is completed in 4 years; most other dwarf mistletoe species complete their life cycle in 5–6 years (Baranyay and Smith 1977; B.C. Ministry of Forests 1983). Hemlock dwarf mistletoe is one the fastest spreading dwarf mistletoes in British Columbia (Baranyay and Smith 1977; Smith 1977; B.C. Ministry of Forests 1983; Smith 1985).

Swelling occurs by the second to third year after hemlock dwarf mistletoe seed infection. Aerial shoots are formed at the end of the second year or in the third year. Flowers are formed in the third and fourth year. Most *A. tsugense* seeds land 2–4 m from

the source. Seeds can land on the crown or seeds can miss the tree completely (Smith 1977; B.C. Ministry of Forests 1983; Smith 1985).

Meiosis occurs in July for hemlock dwarf mistletoe. Male and female flowers occur in July to September with the peak of the flowering occurring in August. Maturation period for mistletoe fruit averages 13–14 months. Seed dispersal for *A. tsugense* occurs in late September to early November. Seed germination is usually from January to May in British Columbia (B.C. Ministry of Forests 1983; Smith et al. 1993; Hawksworth and Wiens 1996; Geils and Hawksworth 2002; Wass and Mathiasen 2003).

Germination and infection percentages of dwarf mistletoe seeds vary from year to year. The average infection rate can vary from 16 to 45%. High mortality of hemlock dwarf mistletoe seeds can occur in some years due to fungal and insect predators (Smith 1977).

1.5.6 Detection and Description of Western Hemlock Dwarf Mistletoe (*A. tsugense* spp. *tsugense)*

The first indication of hemlock dwarf mistletoe is a fusiform, localized swelling on a branch. Aerial shoot heights range from 5 to 13 cm (mean 7.8); colour is greenish to reddish, darker in the winter. Basal diameter of dominant shoots is 1.5–7.0 mm (mean 2.0) (B.C. Ministry of Forests 1983; Hennon et al. 2001; Geils and Hawksworth 2002; Wass and Mathiasen 2003). The shoots may be sparse and poorly developed or not present in dense stands, older infections, and lower branches. Shoots reach their maximum height on open-grown trees or in upper and middle canopy (Shaw and Weiss 2000). Witches' brooms vary from small, in young or new infections, to large palm-like structures with masses of branches that can be 100 years old (Hawksworth and Wiens 1996; Hennon et al. 2001; Wass and Mathiasen 2003).

Male flowers are characterized as 3.6 mm across; a perianth 3- or 4-merous; and shoot segments average 1.2 mm long and 1.0 mm wide and a mean anther diameter of 0.5 mm. Female flowers are characterized as 1 mm across; mature fruit 3 × 2 mm with the proximal portion average 2.0 mm long (Hawksworth and Weins 1996; Geils and Hawksworth 2002; Wass and Mathiasen 2003). Hemlock mistletoe colour is yellow-green

to yellow-purple (Hawksworth and Wiens 1996; Geils and Hawksworth 2002; Wass and Mathiasen 2003).

1.5.7 Damage of Hemlock Dwarf Mistletoe (*A. tsugense* spp. *tsugense*)

The presence of the endophytic system in the hemlock's wood alters its physical and chemical properties resulting in reduced wood quality. Large knots in the hemlock associated with brooms, located on the stem, also reduce wood quality. Severely infected stands increase the opportunity for catastrophic outbreaks of insects, diseases, and fires (B.C. Ministry of Forests 1983; Muir 2003).

Hemlock dwarf mistletoe can reduce the growth of the hemlock tree. In trees with more than 50% of the branches that are infected or a DMR of 3 or more, growth rates were estimated to be reduced by 20–40% compared with uninfected hemlock trees (Smith 1969). Smith (1969) compared a lightly infected hemlock (DMR 1–2) trees with severely infected (DMR $\geq$3) hemlock trees for 7 years. At 15 inches (38 cm) breast height diameter, the lightly infected trees were 14 feet (4.3 m) taller and 14 cubic feet (0.4 cubic meters) greater in volume than severely infected trees (Smith 1985).

1.5.8 Management of Hemlock Dwarf Mistletoe (*A. tsugense* spp. *tsugense*)

Several difficulties can be encountered in a survey for hemlock dwarf mistletoe. Symptoms of dwarf mistletoe aerial shoots, individual witches' brooms, and branch infections are often difficult to discern especially in upper crowns or dense stands (Smith 1977).

Tinnin (1998) suggests a variation to the DMR system called the BVR or broom volume rating. BVR is calculated by determining the volume of brooms within the top, middle, and bottom third of the crown. The rating system is based on how much of the partitioned crown is occupied with a broom. If there are no infections in a crown third, it is scored as zero. If one or more infections occupy less than half the volume of the crown, it is scored as 1. If brooms occupy more than half the crown, it is scored as a 2. The crowns thirds are summed, and the total value is determined. BVR ranges from 0 to 6 and can be estimated quickly. In most cases, BVR will be comparable to DMR. BRV is an effective alternative method for rating dwarf mistletoe, especially in situations that

produce large, dense brooms that make counting branches difficult when using the DMR system (Tinnin 1998).

Most of the silviculture methods used to control dwarf mistletoe can be applied to hemlock dwarf mistletoe (Section 1.3). Healthy hemlock trees have dense foliage and on good plantation sites hemlock trees can outgrow the hemlock dwarf mistletoe, as discussed in hemlock dwarf mistletoe biology (Richardson and van der Kamp 1972; van der Kamp 1998; Shaw and Weiss 2000). Factors such as site condition, location of site (elevation), mix of trees in the stand, level of dwarf mistletoe infection, and spacing of the host trees are important for determining the method of timber harvest. One the most important factors to consider with hemlock dwarf mistletoe infected trees are the method of harvest and change in light levels to the remaining infected trees (Richardson and van der Kamp 1972; Mathiasen 1996; van der Kamp 1998; Shaw and Weiss 2000).

Variable retention harvesting in old, infected forests opens the stand, greatly increasing the spread and intensification of hemlock mistletoe infections. Increased light levels can rejuvenate dominant dwarf mistletoe swellings resulting in an increased production of hemlock dwarf mistletoe seed. In retention harvests, the stand should be evaluated to determine the number of large infected trees on the border, determine the infection level in the stand, and determine the spatial distribution of infected and non-infected trees in the stand (Mathiasen 1996; Shaw and Weiss 2000; Muir et al. 2004).

ESSA Technologies Ltd was in the process of developing a computer model to help quantitative impacts with hemlock dwarf mistletoe infestations such as stand density, site indices, latitudes, elevations, and retention silvicultural systems. This model will also be used to project the effectiveness of alternative management practices such as biological control agents (Robinson et al. 2000; Robinson et al. 2002) (Section 1.3.1).

1.6   Hyperparasites of Hemlock Dwarf Mistletoe: *C. gloeosporioides* as a Biological Control Agent

The known hyperparasites of *A. tsugense* include *C. gloeosporioides*, *Cylindrocarpon gillii*, and *Neonectria neomacrospora* (Anamorph *Cylindrocarpon cylindroides*) (Muir 1967; Shamoun 1998; Kope and Shamoun 2000; Shamoun 2003). Recent work has included the investigation of *N. neomacrospora* and *C. gloeosporioides* as biological

control agents for hemlock dwarf mistletoe (Shamoun et al. 2003; Rietman 2004; Ramsfield et al. 2005).

C. gloeosporioides is one of the most used biological control agents in both forestry and agriculture (Bailey and Jeger 1992). Table 1.3 demonstrates some examples of *Colletotrichum* biological control agents used in both agriculture and forestry. Nineteen *Colletotrichum* strains have been used for the biological control of weeds. *Colletotrichum* species have been used to control a number of different weeds and other pests (Bailey and Jeger 1992; Butt 2000)

Table 1-3 Examples of biological control agents with *Colletotrichum* spp adapted from Butt, 2000)

Common name	Target weed	Causal agent	Crops infested and area	Company or agent
Collego	Northern jointvetch	*C. gloeosporioides* f. sp. *aeschymomene*	Rice and soybean	Ecogen, Inc
BioMal	Round-leaved mallow	*C. gloeosporioides* f. sp. *malvae* (Penz.) Penz. and Sacc.	Field crops	PhilomBios
Burr anthrancose	*Xanthium spinosum* (LINN.)	*C. xanthii* Halst.	Rangeland, Austria	Bruce Auld
Velgo	*Abutilon theophrasti* Medic. (Velvet leaf)	*C. coccodes* (Wallr.) S. Hughes	Lima beans	Alan K. Watson
Lubao 1	*Dodder*	*C. gloeosporioides* f. sp. *cuscutae* T.-Y. Zhang	Field crops	Dr. Li Yang-han
Cassia	*Cassia occidentalis* L. (coffee senna)	*Colletotrichum dematium* (Pers:Fr) Grove	Pasture, Hawaii	Dr. Gadauska
Sida	*Sida spinosa* L. (prickly sida)	*Colletotrichum malvarium* (Braun and Casp) Southw.	Cotton, soybean	Kirkpatrick

The recent move to retention harvesting and restrictions in harvesting in riparian reserves limit the opportunities for silvicultural control of dwarf mistletoe. Alternative methods of control such as the use of biological agents may be better suited for variable retention regimes. *C. gloeosporioides*, commonly isolated from dwarf mistletoes in

western Canada and the United States, has been destructive on both *A. americanum* and *A. tsugense* in western Canada (Muir 1967, 1977; Kope 1997; Ramsfield 2002; Ramsfield et al. 2005). For this study the use of the native parasitic fungus *Colletotrichum gloeosporioides* (Penz). Penz & Sacc. was studied to determine its potential as a biological control agent for western

1.6.1 Pathogenesis of *Colletotrichum* Species

The two main sources of inoculum are conidia produced in acervuli, and ascospores produced in and released from the perithecia for *Colletotrichum* species (Bailey and Jeger 1992; Agrios 1998). In some species, perithecia and ascospores are occasionally produced but in most species acervuli are the main source of inoculum (Agrios 1988; Bailey and Jeger 1992). In the case of western hemlock *C. gloeosporioides*, no teleomorph has been discovered. In young acervuli, conidia are encapsulated in a moist complex structure comprised of polysaccharides, glycoprotein, and a minor mixture of enzymes (Bailey and Jeger 1992; Perfect et al. 1999). This spore matrix is a moist hydrophilic mucilaginous material. As the productive structures mature, the matrix dries and becomes a "crusty" deposit, which binds the spores together. The release of the conidia in young acervuli occurs by water droplet (Bailey and Jeger 1992; Perfect et al. 1999). The conidia of the *Colletotrichum* species are dispersed by rain splash and they rapidly adhere to the aerial parts of the host plant. With perithecia and older acervuli, wind distributes the dry spore masses (Agrios 1988; Bailey and Jeger 1992).

The spore matrix is critical for conidia and ascospore survival (Nicholson and Moraes 1980; Nicholson et al. 1992; Bailey and Jeger 1992). Laboratory experiments have shown that the matrix inhibits spore germination. Inhibitors such as gloeosporone have been found in the spore matrix. These inhibitors remain with the acervuli until water droplets dilute the spore matrix ensuring the distribution of the conidia and preventing premature spore germination (Lax et al. 1985; McRae and Stevens 1990; Bailey et al. 1992). The spore matrix is also important in maintaining and protecting the viability of the spores under low humidity, extreme temperatures, and ultraviolet light, and from the effects of toxic plant metabolites. Self-inhibitors of conidia germination on plant surfaces are

present on the matrix when large numbers of conidia are present (Bailey and Jeger 1992; Morin et al. 1996).

The enzymes in the spore matrix, such as invertase, polygalacturonase, cellulase, and pectin lyases, are thought to be involved in the nutrition during the germination to facilitate the penetration of the plant surfaces or subsequent growth within tissues (McRae and Stevens 1990; Bailey and Jeger 1992).

For *Colletotrichum* to penetrate the plant, it is essential that the spores attach to the plant surface. No clear evidence can determine the nature and origin (from the conidia or spore matrix) of these adhering substances (McRae and Stevens 1990; Perfect et al. 1999). The chemical and physical properties of the conidial matrix of the *Colletotrichum* have been implicated in several parts of the infection process including adhesion of inoculum to the host surface, host recognition, modification of the chemical environment on the host surface, spore germination, appressorial formation, and host penetration (Bergstrom and Nicholson 1999).

After the spores adhere to the plant surface, the conidia and ascospores germinate and undergo a differentiation by forming an appressoria. Germination of spores can be triggered by both physical and chemical signals from the host surface (Bailey and Jeger 1992; Kuo 1999). Selective triggers such as surface waxes and ethylene may induce appressoria formation. Such chemical triggers are thought to make *Colletotrichum* species specific to one host species (Bailey et al. 1992; Wharton et al. 2004). Appressoria are essential for host infection and are generally surrounded by a slimy matrix that is thought to play a role in adhesion and protection of the appressoria (Bailey and Jeger 1992; Kuo 1999; Perfect et al. 1999).

Melanization of the appressoria of *Colletotrichum* is essential for successful penetration of the fungus directly through the epidermis (Morin et al. 1996; Kuo 1999; Perfect et al. 1999; Estrada et al. 2000). Melanin is deposited in a layer of the *Colletotrichum* appressorium cell wall close to the plasma membrane. The *Colletotrichum* genus has developed specialized structures for infection, which include an appressorium germ tube, an intra- or intercellular hyphae, and secondary necrotrophic hyphae (Morin et al. 1996; Perfect et al. 1999; Kuo 1999). Maturation of the appressorium involves formation of a penetration pore in the base of the cell, deposition

of new cell wall layers, and the secretion of extra-cellular matrix (composed of glycoproteins) materials (Bailey and Jeger 1992; Perfect et al. 1999; Kuo 1999). In some species of *Colletotrichum*, the penetration pore becomes surrounded by a funnel-shaped structure called a cone. This cone appears to be an extension of the penetration peg wall and may focus hydrostatic pressure to the site of penetration. In other species, appressorial cones are not present but the cell wall forms a thickened ring around the penetration pore (Perfect et al. 1999). Apical growth resumes with the emergence of the penetration peg through the pore (Bailey and Jeger 1992; Perfect et al. 1999).

Most *Colletotrichum* species can penetrate cuticles directly by formation of appressoria. The three main theories on *Colletotrichum* appressorium penetration are that penetration into host epidermis is achieved by (1) mechanical force alone, (2) the secretion of cutinase degrading enzyme alone, or (3) a combination of both (Bailey and Jeger 1992; Kuo 1999; Perfect et al. 1999). Infection or penetration of the host can occur through (1) wounds caused by insects or physical damage, (2) penetration of the cuticle and epidermis cell wall, and (3) infection in the subcuticular regions or between cells (Bailey and Jeger 1992; Kuo 1999; Perfect et al. 1999).

Colletotrichum gloeosporioides exhibits a two-phase infection process involving an initial symptomless or biotrophic phase, during which the pathogen establishes itself in the host tissues, followed by a destructive phase (Bailey and Jeger 1992; Wharton et al. 2004). During the biotrophic phase, some of these pathogens invade the host cells without killing them. The second phase (necrotrophic phase) of infection occurs after the pathogen is established in the host and the pathogen kills the host cells. This two-phase infection process is called hemibiotrophic or facultative biotrophy (Perfect et al. 1999; Goodwin 2001; Wharton et al. 2004).

The biotrophic infection stage of *Colletotrichum* results in no host disease symptoms and nutrients for *Colletotrichum* are obtained from the living cells. *Colletotrichum* species use two infection biotrophy strategies (Bailey and Jeger 1992; Goodwin 2001). The genus *Colletotrichum* uses an intracellular or subcuticular biotrophy. The initial stages of infection for both biotrophy groups include conidia adhering to the host, germination on the host plant surfaces, production of germ tubes, and formation of the appressorium that will penetrate the epidermis of the host tissue (Perfect et al. 1999;

Goodwin 2001). Following penetration, a sub-epidermal biotrophic pathogen develops beneath the cuticle by forming an intramural network of hyphae, before spreading rapidly throughout the tissue with both inter or intracellular hyphae, killing in advance of the mycelial spread (Bailey and Jeger 1992; Perfect et al. 1999; Goodwin 2001; Wharton et al. 2004). Most *Colletotrichum* species exhibit intracellular colonization. Following penetration, hyphae grow within the cell lumen without penetrating host protoplast. These pathogens grow between plant plasma membranes and plant cell walls (Perfect et al. 1999; Goodwin 2001; Wharton et al. 2004).

The intracellular biotrophy strategy may require a specific metabolic interaction with the hosts, thus limiting the host range (Wei et al. 1996; Wharton et al. 2004). *Colletotrichum* species that have an initial subcuticular growth phase appear to attack a wide range of unrelated species (Wei et al. 1996). Host range is an important characteristic for a biological control. This characteristic may be a useful screening tool for selecting *Colletotrichum* isolates with narrow host ranges (Wei et al. 1996). During the intercuticular colonization, the host plant does not appear to recognize the pathogen and there is no specific resistance response. *C. gloeosporioides* has been known to infect its hosts intracellularly (e.g., *C. gloeosporioides* infects *Stylosanthes* spp.), intercuticularly (e.g., *C. gloeosporioides* infect *Carica papaya*), and some *C. gloeosporioides* can infect both intracellularly and intercuticularly (e.g., both methods of infection are used by *C. gloeosporioides* that infects *Citrus* spp.) (Bailey and Jeger 1992; Wei et al. 1997).

After *Colletotrichum* has successfully colonized the plant tissues, the pathogen growth switches to "classic" necrotrophic behaviour which is responsible for the anthracose and blight symptoms that are typical of the *Colletotrichum* disease (Bailey and Jeger 1992; Wharton et al. 2004). During this phase the pathogen grows extensively throughout the host tissue, inside cells, in walls, throughout walls, and in intercellular spaces. Necrotrophy results in the increased expression of plant cell wall degrading enzymes such as endopolygalacturonase and pectin lyases (Perfect et al. 1999; Wharton et al. 2004). Nutrients are obtained from dead host cells that have been killed by the fungus. The degradation of the plant cell walls allows for the infection to spread in the host

(Bailey et al. 1992; Bailey and Jeger 1992). Production of acervuli or perithecia on plant surfaces appears to require an intact epidermis (Agrios 1988; Bailey and Jeger 1992).

Most fungal spores require exact moisture and temperature conditions for germination and host penetration (Estrada et al. 2000; Wharton et al. 2004). These conditions may not frequently occur under field conditions and can result in an ineffective biological control application (Green et al. 1997). Two major conditions must occur to allow the biological control agent to penetrate the host tissue: (1) the fungal spore must adhere to the surface of the dwarf mistletoe shoots and (2) the dew must be adequate (or free water present) to allow for spore germination. Formulation is important in overcoming environmental variability (Bailey and Jeger 1992; Green et al. 1997).

1.6.2 Taxonomy of *C. gloeosporioides*

Colletotrichum is a large genus and is distributed worldwide. It is a common causal agent of anthracnose, the black, sunken lenticular necrotic lesions contained in acervuli that infect the organs of their host species (Bailey and Jeger 1992). In 1957, von Arx revised the taxonomy of the genus from 750 species of *Colletotrichum* to 11 taxa based on morphology rather than on host specificity. The number of accepted species has now increased to 39, based on morphology, cultural characteristics, and pathology (Sutton 1990, 1992; Bailey and Jeger 1992). The teleomorph of *C. gloeosporioides* is *Glomerella cingulata* (Stonem.) Spauld & Schrenk. Presently, 120 different species for both *Colletotrichum* and *Glomerella* are known (Bailey and Jeger 1992; Sutton 1992).

C. gloeosporioides is classified as a Deuteromycete or a Coelomycete, since the teleomorph is rare and is difficult to induce (Sutton 1980). Deuteromycetes are fungi that are classified based on the asexual stage of the fungi. Most fungi belonging to this group do not have a teleomorph stage. Deuteromycetes are subdivided into three classes: Hypomycetes, Coelomycetes, and Agonomycetes (mycelia sterile) (Sutton 1980, 1992). Coelomycetes conidia are formed within a cavity lined by fungal tissue, host tissue, or a combination of both. A number of different morphological characteristics are used for the classification for *C. gloeosporioides* within the Deuteromycetes (Sutton 1990, 1992). Sutton's classification (1992) is based on the type of conidiogenesis, conidia shape,

conidiophore, conidiostroma, and other specialized structures such as pycnidia, appressorium, and acervuli.

C. gloeosporioides belongs to the order Coelomycetes, which consists of parasites and saprobes of terrestrial vascular plants inhabiting twigs, branches, and leaves of various plant hosts. These fungi are asexual fungi that produce their reproductive propagules in fruiting bodies known as conidiomata (Sutton 1980, 1992). In contrast, the sexual, meiotic Ascomycetes, known as ascomata, contain sexual ascospores enclosed in asci (Agrios 1988). Some species of Coelomycetes have a teleomorph phase in their life cycle and are usually connected to various genera of Ascomycetes (Sutton 1980; Bailey and Jeger 1992).

C. gloeosporioides produces conidia in acervuli, which places it in the order Melanconiales (Sutton 1980; Sutton 1992). The acervuli of *C. gloeosporioides* lack lateral and upper walls to the basal stroma, and there is no specialized method of dehiscence of the fruiting body (Sutton 1980; Sutton 1992). With the *C. gloeosporioides* that attacks hemlock dwarf mistletoe the teleomorph has not be found.

Acervuli are immersed and may be separate or confluent. Conidiogenous cells formed on the inner surface of the walls are blastic (blown out) (Sutton 1992).

C. gloeosporioides infections on western hemlock dwarf mistletoe first appear as small, black to brown necrotic lesions, on the shoots or nodes of the berries. Lesions enlarge and coalesce, and cause dieback and death of the shoots. Diseased mistletoe shoots and berries may contain numerous acervuli. Figure 1.7 is a photo that shows acervuli on disease dwarf mistletoe shoot (under a dissecting microscope 10X). These shoots become shriveled and often become black to grey. Under moist conditions, salmon-coloured spores exude from the acervuli on infected shoots and berries (Kope 1997; Kope and Shamoun 2000).

On potato dextrose agar, *C. gloeosporioides* colonies vary from grayish white to dark grey; the aerial mycelium is even and felted or in tufts associated with conidiomata. On the reverse of the petri plate an uneven white to grey occurs and colours darken with age. Figure 1.9 is a photo of *C. gloeosporioides* grown on PDA. Mycelial growth for *C. gloeosporioides* can occur between 10 and 30°C, with optimum growth at 20°C.

Table 1.4 summarizes the microscopic morphology used for the *C. gloeosporioides*
identification. Figure 1.6 is photo of *C. gloeosporioides* on a diseased dwarf berry. Setae
may be present or absent and scelerotia are absent with no ascomata formed in culture.
Appressoria are clavate, ovate, and obovate and the average size is 6–20 × 4–12 μm.
Conidiogenous cells are enteroblastic, phialidic, hyaline, and cylindric, and borne on
short conidiophores that arise from acervuli. Conidia are formed in pale salmon masses)
that are straight, cylindrical, apex obtuse, and base truncate; their average size is 12–17 ×
3.6–6 μm (Sutton 1980; Sutton 1992; Kope 1997; Kope and Shamoun 2000). Figure 1.8
shows the conidia of *C. gloeosporioides* using a compound microscope (100 μm).

The teleomorph or perfect stage of *C. gloeosporioides* is *Glomerella cingulata*
(Stonem.) Spauld & Schrenk. *G. cingulata* has either single or clustered immersed,
subhyaline to brown, subglobose perithecia that average 100–350 μm in diameter. The
ostioles of perithecia are appellate or beaked with external hairs. Asci are cylindrical to
ellipsoid with 4–8 spores averaging 35–80 × 8–14 μm. Ascospores are hyaline and
ellipsoid in shape; ascospores are unicellular with dense granular cytoplasm at the poles
averaging 12–28 × 4–7 μm (Sutton 1980, 1992). The perfect phase of *C. gloeosporioides*
has not been reported on *A. tsugense* (Kope 1997; Kope and Shamoun 2000).

Molecular or DNA-based methodology can provide a valuable tool for taxonomic
information on *Colletotrichum* species. Sequence analysis is useful within the large
ribosomal subunit and is used in distinguishing closely related taxa. In a large number of
fungi, sequence comparisons in two regions of the large ribosomal subunit, subunit
domain 2 and the internal transcribed spacers (ITS-1 and ITS-2) show the percent
sequence variation in a range useful for phylogenetics comparisons (Nichole et al. 1997;
Hillis et al. 1991). The ITS 1 sequences were used to distinguish the different species
within *Colletotrichum* and for inferring phylogenetics relationships between species. For
differentiating between *C. gloeosporioides* and *C. acutatum* Simmonds ex Simmonds,
nucleotide sequencing data were obtained from a portion of the mitochondria small
subunit rDNA (mtSSU) and a fragment of the B-tubulin gene in combination with ITS
sequencing. Using this sequence can assist in the screening of large numbers of
Colletotrichum isolates and assure reliable identification to the species level (Vinnere
2004; Wharton et al. 2004).

In conventional classification the species or groups of species of *Colletotrichum* is differentiated based on morphology, growth physiology, or soluble protein banding patterns. The recognition of sub-specific taxa, particularly at the forma specialis, race, and biotype levels, is still controversial. It is also important for the standardization of protocols by which the organisms are placed in taxonomic groups or genera (Sutton 1992; Wharton et al. 2004). This includes standardized methods of media and conditions of growth, physiology, and primary metabolism (such as temperature relationships, pH, growth, growth in the presence of various compounds, and primary biochemistry). At the moment there are no internationally accepted conventions (Sutton 1992; Wharton et al. 2004). The study of the taxonomy of *Colletotrichum* is in its infancy for molecular biological techniques. In the future, molecular data may be correlated with morphological comparisons to be used to determine taxonomic relationships within a genus. Molecular and conventional taxonomy could easily group these fungi into their sub-specific taxa (Bailey and Jeger 1992; Wharton et al. 2004).

Table 1-4 Microscopic description of *Colletotrichum gloeosporioides*

	Colletotrichum gloeosporioides
Acervuli	On both sides of leaf, non-seta.
Conidiophores	Conidiogenous cells enteroblastic, phialidic, hyaline, cylindric conidiophores that arise from upper acervuli.
Conidia	Conidia hyaline, cylindrical to slightly clavate, non-septate, 12–20 × 4–5 um. Salmon coloured on mass.
Colonies on PDA	Grayish to blackish, some darken in colour with age.
Appressorium	Brown, clavate to irregular in edge, 9–24 × 4–12 um in size.
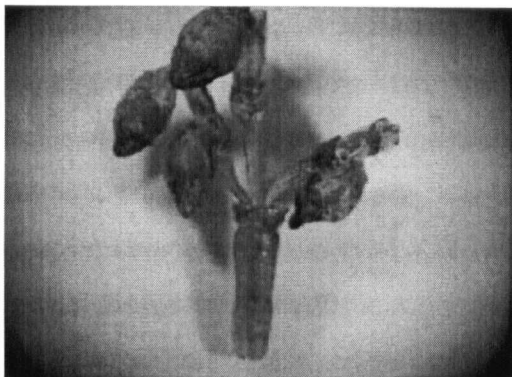
Figure 1-6 Diseased dwarf mistletoe shoot and berry-

Figure 1-7 Acervuli on a dwarf mistletoe shoot- 10 µm
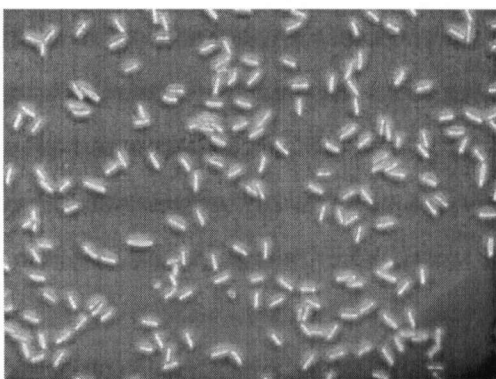
Figure 1-8 *C. gloeosporioides* conidia-(100 µm)
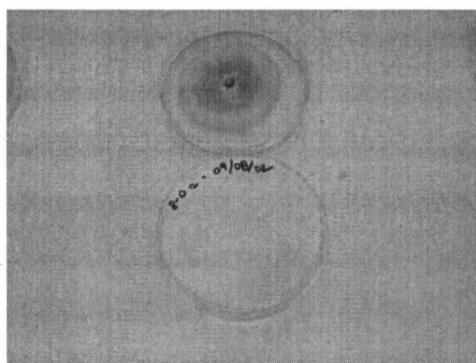
Figure 1-9 *C. gloeosporioides* grown on potato dextrose agar

1.6.3   Life cycle of *C. gloeosporioides*

The life cycle of *C. gloeosporioides* that infects dwarf mistletoe plants is based on asexual spores or conidia. The acervuli consist of a cushion of mycelia, called a stroma, several layers thick, and develop just below the cuticle, which is ruptured by the upward pressure of the developing mass of conidiophores and conidia. The conidia are held together in a sticky mass, which is firm and crusty in dry conditions, and may be washed or spattered by raindrops or carried by windblown mist and insects (Agrios 1988).

Conidia germinate when there is free moisture on the leaf or fruit structures. Upon germination, a conidium produces a hyphal strand or mycelium, which forms an appressorium (Agrios 1988; Bailey and Jeger 1992; Wharton et al. 2004). From the appressorium, hyphae penetrate the leaf epidermis (Kuo 1999). Infection of dwarf mistletoe is by direct penetration of uninjured tissue by conidia (Agrios 1988; Bailey and Jeger 992). Early infection and invasion of host tissue results in intercellular mycelial

growth that remains latent for some time before *Colletotrichum* invades the host cells and the host tissue collapses and rots (Agrios 1988). The lesion appears as a small, dark, pinpoint-size spot, which quickly expands. The mycelium then produces acervuli and conidia just below the cuticle, which will rupture and release conidia that will cause more infections (Sutton 1992; Agrios 1988). The fungus can attack young leaves, stems, and fruit, and can remain latent until environmental conditions become favourable or when the tissues mature at which point the infections develop fully. Conidia are generally spread by rain. The fungus can overwinter in diseased stems, leaves, and fruit as mycelia or spores (Bailey and Jeger 1992; Wharton et al. 2004). The surviving mycelia quickly produce conidia in the spring that can cause primary infections and continuous secondary infections during the entire season as long as the temperature and humidity are favourable (Agrios 1988).

1.7   Research objectives

The purpose of this thesis was to evaluate *C. gloeosporioides* as an inundative biological control agent for *A. tsugense*. The specific objectives were:

1. To select a lead isolate of *C. gloeosporioides* based on virulence test on detached dwarf mistletoe swellings.

2. To determine the best substrate for the mass production of *C. gloeosporioides*.

3. To determine the optimum growth and conidia germinations temperatures of *C. gloeosporioides*.

4. To determine the efficacy in field conditions of two *C. gloeosporioides* formulations applied to dwarf mistletoe swelling.

5. To determine the impact of *C. gloeosporioides* on dwarf mistletoe shoots and berries under field conditions.

2 CHAPTER 2.0- SELECTION OF AN ISOLATE OF *C. GLOEOSPORIOIDES*

2.1 Introduction

The first step in developing an inundative control is to screen potential *C. gloeosporioides* isolates for virulence and temperature response for both mycelial growth and conidia germination (Templeton et al. 1986). Optimum temperature ranges for the hemlock *C. gloeosporioides* isolates for both mycelial growth and conidia germination *in vitro* may allow for a better understanding of *C. gloeosporioides* biology. This information can help establish the best timing of the application of *C. gloeosporioides* to the *A. tsugense* swellings to maximize the infection rate (Templeton 1982; Templeton et al. 1986; Ramsfield 2002).

Greenhouse studies can also determine preferred environments for spore germination, pathogenesis, and incubation periods for this biological control agent (Parmeter et al. 1959; Templeton 1982; Templeton et al. 1986). Unfortunately, greenhouse studies were not conducted since the hemlock seedlings inoculated with *A. tsugense* seeds in spring of 2002 did not develop mature *A. tsugense* shoots in time. Alternative, *in vitro* experimental units were developed to test for virulence.

The objectives for this chapter are to select a lead *C. gloeosporioides* isolate for a subsequent field trial. This will include the following steps:

1. Assemble a set of *C. gloeosporioides* isolates derived from hemlock dwarf mistletoe from dispersed geographic locations.
2. To determine the best substrate for production of *C. gloeosporioides* conidia.
3. To eliminate isolates of *C. gloeosporioides* that do not produce abundant conidia, if any.
4. To develop an *in vitro* pathogenicity test for *C. gloeosporioides* on dwarf mistletoe and test the set of remaining isolates.
5. To assess *in vitro* pathogenicity and eliminate isolates of low pathogenicity.
6. To determine temperature ranges and optimum temperatures for both linear mycelial growth and conidia germination for the remaining isolates.
7. To screen and monitor isolates applied in a small field site located at Pacific Forestry Centre for a 1 year.

2.2 Materials and Methods

2.2.1 Collection of *C. gloeosporioides* isolates and testing conidia substrates

Diseased *A. tsugense* shoots, fruits and swellings were collected from different locations from Vancouver Island and these cultures were maintained in a PFC culture collection. In the summer of 2001, diseased *A. tsugense* shoots and berries were collected from Holt Creek area near Cowichan Valley on Vancouver Island. Table 2.1 describes the geographical origin of the *C. gloeosporioides* isolates screened in this study. These samples were surface sterilized by placing the diseased materials in 95 % ethanol for 2 minutes, followed by placing the parts in 10% sodium hypochlorite for 2 minutes, followed by 3 2-minute washes of sterile distilled water. The surface sterilized pieces were then transferred to potato dextrose agar (PDA, Difco Laboratories, Detroit, USA) and incubated for approximately 10 days at 20°C.

Table 2-1 Geographical origin of *C. gloeosporioides* isolates examined and described in this study.

PFC isolate #	Location	Area	Collection Date	GPS (Site)
2415	Spider Lake	Parksville,	09/13/96	$49^0 21' 00''$ N $124^0 38'$, $00''$ W
2280	Bowser	Parksville,	09/25/97	$49^0 26' 00''$ N $124^0 03' 00''$ W
4060	Holt Creek	Cowichan	07/19/01	$48^0 45' 00''$ N $123^0 50'$, $00''$ W.
4058	Holt Creek	Cowichan,	07/19/01	$48^0 25' 00''$ N $123^0 50'$, $00''$ W
4059	Holt Creek	Cowichan,	07/19/01	$48^0 45' 00''$ N $123^0 38'$, $00''$ W
Strawberry isolate	Abbotsford	Fraser Valley	?	SFU collection

- **Longitudes and latitudes were cross referenced with BC Ministry of Highways, BC Maps (2001).**

Hyphae that emerged from the dwarf mistletoe material were subcultured from the hyphae tips (using aseptic techniques). Hyphae tips were transferred to new PDA plates and incubated at 20°C. New colonies were then identified based on morphological

characteristics using a compound microscope. For long-term storage, cultures were placed in 10 % glycerol solution and placed in an -80 ° C freezer.

The PFC isolates all originated from dwarf mistletoe shoots collected on Vancouver Island area suggesting these isolates may have similar growth and conidia germination rates. A strawberry isolate of *C. gloeosporioides* originating from strawberry fields from Fraser Valley was obtained from the Simon Fraser University, Plant Pathology Department culture collection. The strawberry isolated used to compare and differentiate subpopulations of *C. gloeosporioides* grown on the basis of temperature for both mycelial growth and conidia germination.

2.2.1.1 Production of *C. gloeosporioides* conidia on different substrates- Substrate selection

The purpose of this trial was to measure the number of spores produced by each isolate on different solid-based media. Four different cereal grains, slow oats, wheat bran, rice and millet were assessed for conidia production and ease of conidia extraction. The spore production of the PFC isolates with two replicates was produced on each cereal grain.

Twenty grams of each cereal grain was added to each 500 mL Erlenmeyer fast. Twenty ml of sterile distilled water was added to millet, and rice, 30 ml of distilled water was added to the slow oats and 15 ml of distilled water to wheat bran. The media and the water were mixed in 500ml Erlenmeyer flasks and autoclaved twice for 20 minutes at 121°C.

Six mycelial PDA plugs measuring 7.5 cm in diameter were added to each flask using the sterile technique. Two replications of each isolate (PFC 2415, PFC 2280, PFC 4058, PFC 4059 and PFC 4060) were cultured on each medium type. Each flask was sterilized in an autoclave for 20 minutes, cooled to room temperature and inoculated with an isolate. The isolates were grown at room temperature (20–23°C) for 7-10 days. Each of the cereal grain cultures was washed with 100-mL sterile distilled water by placing the flasks on a rotary shaker at 50 rpm for 60 minutes. The contents of each flask were poured through sterile cheesecloth into sterile beakers. The filtered conidia were placed in two flacon tubes and centrifuged at 2400 ×g for 30 minutes. The supernant was decanted from the flacon tubes and the conidia were re-suspended in 10 mL of sterile distilled

water. The contents of two falcon tubes originating from the same flask were then combined and stored at 4°C. Conidia were stored 4°C and screened for bacterial contamination by plating a subsample of the spore suspension on PDA and growing it at 30°C. Conidia were used within 2 days of production or discarded.

A 10^{-2} dilution of the stock suspension was made from the stock solution of conidia by adding 10 uL of stock solution into 990 uL of sterile distilled water into a 2-mL tube. The number of conidia in 10 µl suspension for each media was determined using a haemacytometer. Three counts (100 conidia/plate) were used for determining number of conidia for each flask.

2.2.1.2 Conidia extraction for conidia germination, virulence and PFC trial

Conidia extraction followed the same procedure outlined in the Production of *C. gloeosporioides* conidia on a millet substrate for (1) the conidia germination trials, (2) spraying virulence trials, and (3) producing *C. gloeosporioides* formulations for PFC isolates for the PFC trials. For the percentage of conidia germinated within a 24 hour period and the 50% conidia germination trials, the conidia suspension for each flask was adjusted with sterile distilled water to spore concentration of 10^5 spores/mL and spread with a sterilized glass rod onto a 1.5 % water agar plate. For the virulence trial a conidia solution of 10^6 conidia suspension was used for spraying the experimental units. For the PFC field trial, a concentration 10^7 conidia/ml was added to mixture of water absorbent starch (5 grams), unrefined corn oil (5ml), confectioners' powder sucrose and Hi-Sil 233 (PPG Industries Inc. Pittsburg P.A. USA) hydrated silica (7 grams). The mixture was spread 2-3 mm thick in shallow pans and dried in a laminar flow hood for 48 hours. This process of to stabilize and granulate *C. gloeosporioides* is called the 'Stabileze process' described by Quimby et al. 1999.

2.2.2 Developing an *in vitro* screening test

Several methods were tested to determine the best method to screen *C. gloeosporioides* isolates in vitro. The screening methods included the following:

1. Placing a detached *A. tsugense* shoots on a moist paper towel and monitoring the viability of the shoots for a week.

2. Placing a detached *A. tsugense* shoots in a nutrient-saturated rock wool cube and monitoring the viability of shoots for a week. Rockwool cube is made from basalt rock it is heat-treated at high temperatures then spun back together like candy.

3. Placing a detached hemlock branch infected with *A. tsugense* in a saturated rock wool cube and monitoring the shoots for 1 week.

2.2.2.1   Detached dwarf mistletoe test (*in vitro* test)

Virulence was screened on detached hemlock branches infected by hemlock dwarf mistletoe and bearing mistletoe shoots. Male and female samples were collected in early August 2002 at the Spider Lake site. The Spider Lake location is found in the Qualicum area of Vancouver Island on Horne Lake road on Block # 347, Plan #33670 in District #3. The GPS reading on the side was 49' 21' 43" N 124' 37' 42" W at 51 meters elevation. The stand consists of young, regenerated, hemlock infected with hemlock dwarf mistletoe located in the Coastal Douglas-fir zone. The infected hemlock branches were collected lower branches.

Pathogenicity was screened on detached hemlock branches infected by hemlock dwarf mistletoe ranging from 10 to 16 cm in length, stem diameter from 5 to 15 mm with the number of shoots ranging from 2 to 30 and female berries varying from 4 to 35. Male and female samples were collected in the fall at the Spider Lake location.

The detached hemlock branch infected with *A. tsugense* was placed in rock wool cubes that had been pre-soaked overnight in hydroponic solution (2 mL/L of Thrive Alive B-1, Techaflora Plant Products, Vancouver, BC), which was placed in the incubator for 24 hours at 15°C.

Five isolates of hemlock *C. gloeosporioides*, from three different locations on Vancouver Island, were evaluated for pathogenicity (Table 2.1). The conidia for each isolate were produced and harvested as outlined in Methods and Materials 2.2.2.1. A 10^6 conidia/mL of conidia/water suspension was sprayed on *A. tsugense* shoots on the detached swellings until run off. A control treatment consisting of water was sprayed on *A. tsugense* swelling until run off. Four replicates of each isolate were used for assessing pathogenicity. Virulence or pathogenicity is defined as the ability cause disease. Koch's postulates proof of pathogenicity is 1) establish a constant association of organism and

disease symptoms, 2) isolate organism and grow in pure culture, 3) inoculate healthy plant and produce disease symptoms and reisolated the disease organism (Edmonds et al. 2000).

The detached dwarf mistletoe infected hemlock branch was placed in saturated rock wool cube, placed in a germination tray. An equal number of male and female (with berries) dwarf mistletoe swellings were used for each treatment. Excess moisture in the bottom of the germination boxes was removed, and the boxes placed in a Precision® environmental growth (Ohio, USA) chamber at PFC site. The germination tray was placed in incubator for 18 hours at 15°C and 6-hour nights at 15°C. The hydroponic blocks with detached dwarf mistletoe swellings were monitored weekly for 1 month. The treatments included a control and the five different isolates.

Four replicate *A. tsugense* swellings were used for each treatment for testing the pathogenicity. A diseased shoot was defined as any shoot showing symptoms of *C. gloeosporioides*. The percentage of diseased shoots were calculated by dividing the number of disease shoots counted after 4 weeks by the number of healthy shoots counted at the first assessment and multiplying this proportion by 100. The percentage of diseased dwarf mistletoe berries was calculated in the same manner.

2.2.3 Mycelial growth linear growth characteristics of *C. gloeosporioides* at varying temperatures.

The mycelial growth rate of *C. gloeosporioides* was determined for each of the following temperatures 4, 10, 15, 20, 25, and 30°C. PFC isolates 2415, 2280, 4060, 4058, 4059, and a strawberry *C. gloeosporioides* isolate (obtained from Simon Fraser University, Department of Plant Pathology, originated from Abbotsford area) were grown at 6 temperatures to determine mycelial growth rates.

A mycelial plug (7.5 mm) of *C. gloeosporioides* was placed in the centre of a PDA plate (12cm) and incubated at each of the temperatures and for each isolate. Three replicates were used for each of the isolates. The diameter of each colony was measured along two pre-determined lines (a straight line across the centre of plate and the second running perpendicular). The mycelial growth for each plate was the average of the measurements along the two pre-determined lines.

Plates were measured every fourth day for 2 weeks or until the fastest growing colony reached the edge of the PDA plate.

2.2.4   Percentage and rate of conidia germination at varying temperatures

Conidia for each isolate were produced and harvested as outlined in mass production of conidia (Section 2.2.1.2). The conidia suspension for each flask was adjusted with sterile distilled (pH 6.7) water to a concentration of 1.0×10^5 conidia/mL and stored in the fridge for one day.

The germination of the conidia was monitored for each of the five isolates at 4, 10, 15, 20, 25, 30, and 35°C for determining the percentage of conidia germination after 24 hours and in a separate experiment, the number of hours for 50% conidia germination. For each plate 500 uL of the 10^5 conidia/mL suspension were plated on 1.5% water agar plates. Three replicates (each on different plates) for each isolate were counted for each temperature. The percentage of conidia germinated after 24 hours and the 50% conidia germination were determined two separate experiments. Each experiment was conducted at different time periods using the same isolates. One hundred conidia for three different plates were assessed for germination on each plate for percentage of conidia germinated after 24 hours and the 50% conidia germination.

2.2.4.1   Percentage of conidia germination for the PFC isolates after 24 hours

For the percentage of conidia germinated after 24 hours, plates were maintained at 4, 10, 15, 20, 25, 30, and 35 °C and the number of germinated conidia among 100 conidia on each of the three plates were counted at 24 hours.

2.2.4.2   2.2.4.2 Determining the number of hours for 50% conidia germination for each PFC isolate

For the 50% conidia germination test counts were made at 2, 4, 6, 8, 9, 10, 12, and 24 hours were incubated at 4, 10, 15, 20, 25, 30, and 35 °C. Germinated conidia were defined as conidia with a germ tube exceeding the width of the conidia.

The percentage of conidia germinated over time was graphed for each temperature for each isolate. All the isolates that germinated at 4 and 10°C showed polynomial quadratic germination curves while all the isolates that germinated at temperatures between 15 to

35°C showed 3rd parameter sigmoid germination curves. The conidia germination curves for 4 and 10°C were fitted using a polynomial quadratic curve $y = a + b_1x_1 + b_2x_2^2$ where y = percent germination, a= the y intercept or time in hours, b_1 = the slope of percent germination to time b_2= regression constant or coefficients and x = time (hours). To fit a curve for all the isolates germinated at 15–35°C a third parameter sigmoid formula $y = b_1/$ [1+EXP (x–a)/b$_2$ was used. From each graph, the time to reach 50% conidia germination was calculated. The r^2 value for all the 42 graphs varied from 0.899 to 0.997. This indicated a good correlation between x = time (hours) and y = percentage of conidia germinated at a given time. For each temperature the isolates with fast conidia germination will have a lower number of hours for 50% germination than slower germinating isolates. Figure 2.1 is an example of 50% conidia germination curve used to determine the r^2 value.
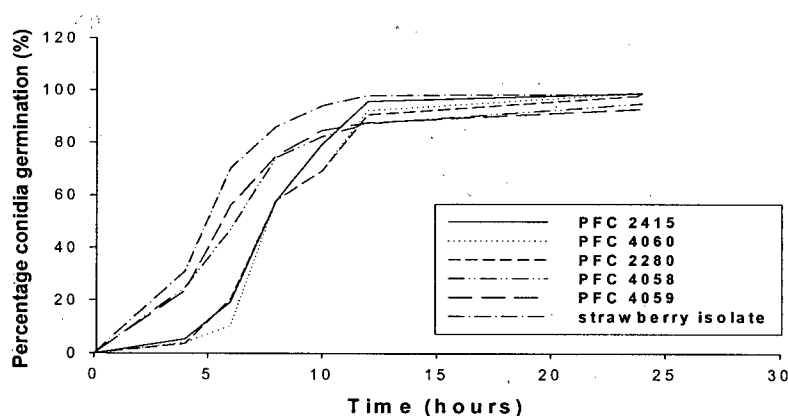
Figure 2-1 Conidia germination curve for all PFC isolates incubated at 20°C. This graph includes percentage conidia germination is plotted against time in hours. These curves are an example of 3rd parametric sigmoid regression line with r^2 = 0.975.

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

At the PFC site in Victoria, B.C., 5- to 6-year-old hemlock trees infected with dwarf mistletoe were used to test pathogenicity of the PFC isolates formulated with 'Stabileze'.

Conidia for each isolate were produced and harvested as outlined Methods and Material 2.2.2.1. A haemacytometer was used to determine the spore concentration. In this case, a spore concentration adjusted to 1.0×10^7 conidia/mL was used in the preparation of a 'Stabileze' formulation.

The 'Stabileze' was formulated by mixing: 5 g of Waterlock (Grain Processing Corporation, USA) water-absorbent starch was mixed with 5 mL of unrefined corn oil (Spectrum Natural, Petaluma, CA). The solution was heated in a microwave oven for 1 minute and cooled to room temperature. Then, 20 mL of the fungal suspension (10^7 conidia/mL) were added to the cooled starch/oil mixture and mixed with a metal spatula. The mixture was allowed to stand to be fully absorbed into the fungal suspension. Twenty grams of Confectioner's powdered sugar (Roger's Sugar, Vancouver BC, Canada) was added followed by 7 g Hi Sil® 233 (PPG Industries Inc., Pittsburgh, PA, USA) hydrated silica. The mixture was thoroughly mixed until small uniform crumbs of about 0.5 cm were formed. The mixture was spread 3–4 mm thick on aluminum tin foil and placed on a plastic tray (38 × 24 cm) and allowed to dry for 48 hours (Quimby et al. 1996; Quimby et al. 1998). Figure 2.2 is a photograph of the final product of Stabileze formulation. Once the mixture was dried, the product was stored in a sealed container at 4°C. For the 'Stabileze' controls, 20 mL of sterile distilled water was added instead of conidia suspension to the cooled starch/oil mixture. The viability of the spores was difficult to determine since the conidia were embedded in the formation. Fresh batch of formulation was used in the field trials.
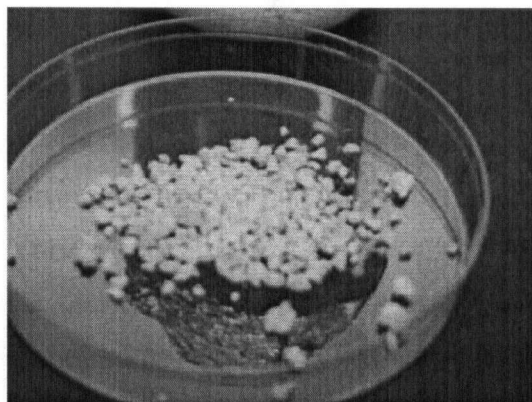
Figure 2-2 Final product of stabileze formulation.

On the day that the treatments were applied, the Stabileze formulation was re-suspended by adding 1 g of the 'Stabileze' mixture to 100 mL of sterile distilled water. The mixture was stirred in a hand shaker and hydrated for at least 30 minutes. Three replicates of each isolate were assigned to separate *A. tsugense* swellings found at the PFC site. The 5 isolates were formulated with 'Stabileze' and applied to each swelling with hand sprayer. The control consisted of distilled water formulated with Stabileze. Each swelling was sprayed until run off. Three swellings per treatment were tagged. Each treatment contained 1 female swelling and 2 male swellings. Treatments were applied at 10 AM on July 31, 2002; the temperature was 24°C during the day, and the relative humidity was 50% with overcast skies (Appendixes I–V).

Assessment of the swellings was conducted biweekly for the first 3 months and then monthly during 1 year. The number of healthy and diseased shoots and berries were counted and digital pictures of each *A. tsugense* swelling were taken at each assessment date. The first assessment occurred 1 week after the treatments was applied. The proportion of diseased shoots and berries was monitored from September 7 to March 14, 2003.

2.2.5.1 Confirmation for the presence of *C. gloeosporioides* on diseased shoots and berries

On October 21, 2002 samples of diseased shoots and berries were collected from PFC field experimental units and aseptically cultured. Culturing the diseased shoots and

berries included surface sterilization in 95% ethanol for 2 minutes, then 10% sodium hypochlorite for 2 minutes followed by 3 washes in sterile water for 2 minutes. The surface sterilized pieces were transferred onto PDA (PDA Difco Laboratories), and incubated for about 10 days before *C. gloeosporioides* colonies were identified. *C. gloeosporioides* colonies were identified based on morphological characteristics such as conidiophores, conidia and mycelial, as described by Sutton 1980; Sutton 1992; and Kope et al. 1998 using a compound microscope.

2.2.6 Statistical analysis

All statistical analyses were performed using Sigma Stat 2.03 (SPSS Inc., Chicago, IL). One-way ANOVAs were used to analyse the number of diseased shoots and berries for each isolate for the detached dwarf mistletoe test and the small outdoor field trial. A two-way ANOVA was used to analyse the production on different substrates for each isolates, mycelial growth, percentage of conidia germinated after 24 hours trial and the number of hours required for 50% conidia germination for each isolate at different temperatures. For tests that did not meet the assumptions of ANOVA (not passing the normality or variance test), a ranked (Kruskal-Wallis) ANOVA was applied to one-way ANOVA tests and ranked (transformed data) ANOVA was applied to two -way ANOVA tests. A repeat measures test was used for analysizing colony diameter over a period of 12 days for 15, 20, and 25°C. Tukey's comparison test was used for parametric data and non-parametric data comparing paired means. The graphs were generated using Sigma Plot 3.1 (Jandel, Sanfael, CA) and Excel® (Microsoft Corporation, USA).

2.3 Results

2.3.1.1 Testing conidia substrates for *A. tsugense C. gloeosporioides* isolates

After autoclaving the four different solid substrates (slow oats, wheat bran, rice, and millet) the millet maintained the best structural integrity, meaning that individual millet grains remained separate whereas the other cereals tended to form a gelatinous mass after autoclaving, greatly reducing the surface area available for spore production. To separate the individual substrate particles for each of the substrates, the media were mixed using a sterilized glass rod. The slow oats, wheat bran, and rice were glutinous and more difficult to work with than the millet.

Figure 2.3 shows the mean number of conidia produced on the four media types. Two-way ANOVA on ranks indicated that the PFC isolates grown on millet produced significantly more conidia than the rice followed by slow oats and wheat bran ($p =$ 0.042). Comparing PFC isolates conidia production on the millet substrate; One-way ANOVA detected that PFC 2415 produced a significant higher number of conidia than PFC 4059 ($p=0.05$). One-way ANOVA detected no significant differences between PFC 2415, PFC 4060, PFC 4058, and PFC 2280 due to large variation of conidia production between the PFC isolates grown on millet substrate.

*I=Standard error of the mean-S.E.

Figure 2-3 Mean numbers of conidia produced on four media types for each fungal isolate of *C. gloeosporioides*.

2.3.2 Developing an *in vitro* test

A number tests were used to evaluate the best method of testing virulence *in vitro*. Both detached dwarf mistletoe shoot method, as outlined in methods and materials 2.2.2, resulted in mistletoe shoots fracturing into stem segments making it impossible to screen the *C. gloeosporioides* isolates for virulence. The hemlock branch infected with dwarf mistletoe placed in saturated nutrient solution remained intact for 4 weeks.

2.3.2.1 Detached dwarf mistletoe test (*in vitro* test)

Five PFC fungal isolates from three different locations on Vancouver Island were screened for pathogenicity (Table 2.1) on detached dwarf mistletoe infected hemlock branches. Four detached dwarf mistletoe infected hemlock branches were sprayed to run off for each PFC isolate.

Table 2.2 shows the disease assessment for the PFC isolates occurred 3 weeks after isolates were applied to the swellings for the virulence test. Statistical analysis showed no significant difference between PFC isolates in the percentage of diseased shoots and berries although PFC 2415 showed the highest percentage of diseased shoots and berries.

Figure 2.4 and 2.5 show control treatment and PFC 2415 treatment respectively, three weeks after the application of the treatments.

Figure 2-4 Control treatment- (This treatment was sprayed with sterile distilled water on detached *A. tsugense* swellings). Three weeks after treatments were applied.
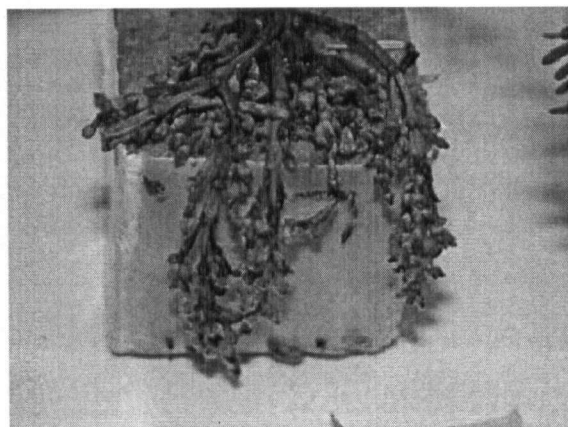
Figure 2-5 PFC 2415 treatment. Three weeks after treatment PFC 2415 has applied to detached *A. tsugense* swelling.

Table 2-2 Percentage of diseased shoots and berries, 4 weeks after the isolates were applied.

PFC isolate #	Location	Number of swellings (replicates)	Total number of shoots	Number of berries	Percentage of disease shoots	Percentage of diseased berries
PFC 2415	Spider Lake, Parksville	4	20	25	60	80
PFC 2280	Bowser, Parksville	4	18	23	56	70
PFC 4060	Holt Creek, Cowichan	4	10	18	30	50
PFC 4058	Holt Creek, Cowichan	4	15	20	40	60
PFC 4059	Holt Creek, Cowichan	4	8	20	50	75
Control	Sterile distilled water spray,	4	12	15	0	0.

Table 2-3 Percentage of *C. gloeosporioides* (*C.g*) isolated and identified from diseased shoots or berries.

Isolate	Number of shoots isolated	Number of berries isolated	Percentage of *C.g.* isolated from shoot	Percentage of *C.g.* isolated from berries
PFC 2415	5	3	100	100
PFC 2280	5	3	80	100
PFC 4060	5	3	60	67
PFC 4058	5	3	80	100
PFC 4059	5	3	80	100
Distilled water (no conidia)	0	0	0	0

- ***Each shoot and berry was plated on one PDA plate. One or more C.g. colony indicated that C.g. was isolated.**

One month after the application of *C. gloeosporioides*, diseased *A. tsugense* shoots and berry samples were collected from detached hemlock swellings to confirm the presence of *C. gloeosporioides*. Diseased samples were aseptically cultured from each isolate to confirm the presence of *C. gloeosporioides*. Table 2.3 summarizes the result of the percentage of *C. gloeosporioides* isolated and identified from diseased shoots and berries.

2.3.3 Linear mycelial growth characteristics of *C. gloeosporioides* at varying temperatures

Figure 2.6 shows the average growth of each isolate at each temperature ranging from 0-30°C. The maximum mycelial growth for PFC 4060, 4059, and 2280 occurred at 25°C (3.3-3.5 mm/day), for PFC 2415 at 20°C (5.0 mm/day) and for PFC 4058 at 15°C (4.1mm/day). The maximum and minimum mycelial growth range for the PFC isolates was between 4-35°C and the optimum growth temperatures for the PFC isolates ranged from 15 - 25°C.

The strawberry isolate *C. gloeosporioides* showed a significantly higher growth rate (p< 0.05) than PFC isolates at all temperatures. The strawberry isolate was included to determine differentiation between different subpopulations of *C. gloeosporioides*.
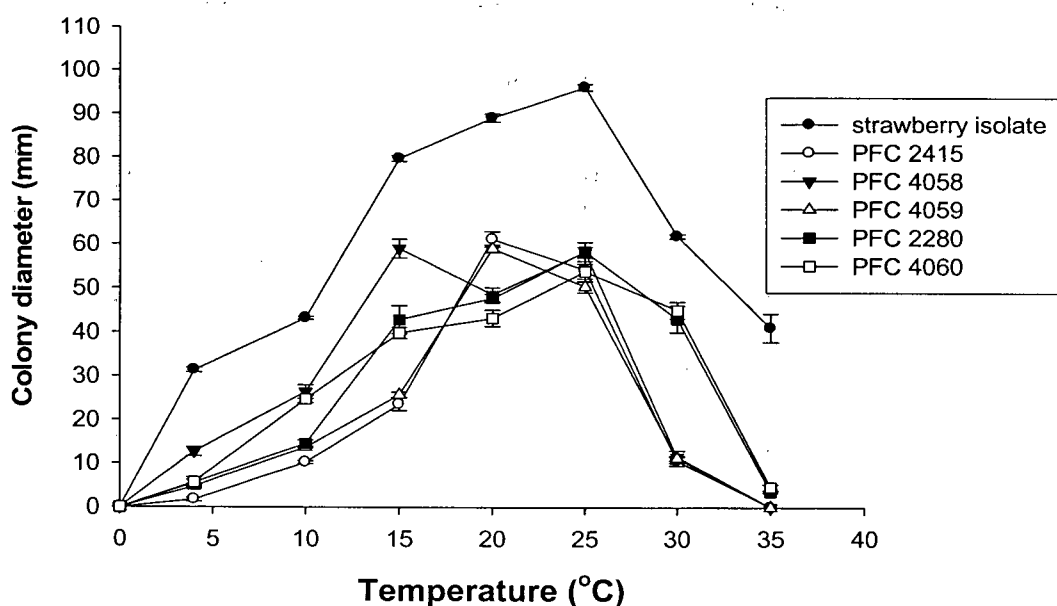
Figure 2-6 Colony diameter of the six *C. gloeosporioides* isolates grown at 4, 10, 15, 20, 25, 30°C after 12 days of incubation.

2.3.3.1 Linear mycelial growth characteristics of the six isolates of *C. gloeosporioides* grown at 15, 20, and 25°C after 12 days of incubation.

Figure 2.7 shows the mycelial growth of the six *C. gloeosporioides* isolates at 15°C after 12 days of growth. A repeated measures ANOVA detected a faster linear mycelial growth rate for PFC 4058 (5.0mm/day) compared to PFC 4060 and PFC 2280 (2.5-3.3 mm/day) while PFC 4059 and 2415 showed a significantly slower linear mycelial growth rate (1.6-1.8 mm/day) than PFC 4060 and 2280 ($p=0.05$) after 12 days of incubation at 15°C. After 8 days of incubation at 15°C, a repeated ANOVA detected a faster linear mycelial growth for PFC 4060 and 2280 compared to PFC 4058 and 2415. After 4 days of incubation at 15°C, a repeated ANOVA for detected a faster linear mycelial growth rate than PFC 4060 compared to PFC 2415, 4058, 2280 and 4059.

Figure 2.8 shows the mean mycelial growth (mm) for six isolates of *C. gloeosporioides* during 12 days of incubation at 20°C. At day 12 days at 20°C, PFC 2415 showed a significantly faster linear mycelial growth rate (5.0 mm/day) compared to PFC 4058, 2280, 4060 and 4059 (3.2-3.5 mm/day). Repeated measures ANOVA revealed faster linear mycelial growth rate for PFC 2415, 4059, 2280 (5 mm/day) ($p=0.05$) compared to PFC 4058 (3.6 mm/day) for mycelial growth at 20°C after 8 days. No significant differences in colony growth were detected at day 4 between the PFC isolates at 20°C.

Figure 2.9 shows the mean mycelial growth (mm) for six isolates of *C. gloeosporioides* during 12 days of incubation at 25°C. For isolates incubated at 25°C after 4 days repeated ANOVA revealed a significant faster linear mycelial growth rate for 4058 and 2415 (5.0 mm/day and 4.5 mm respectively) ($p=0.05$) compared to PFC 2415, 4060 and 4059 (2 -2.5 mm/day). No significant differences were detected in linear mycelial growth between PFC isolates at day 8 and 12 incubated at 25°C.
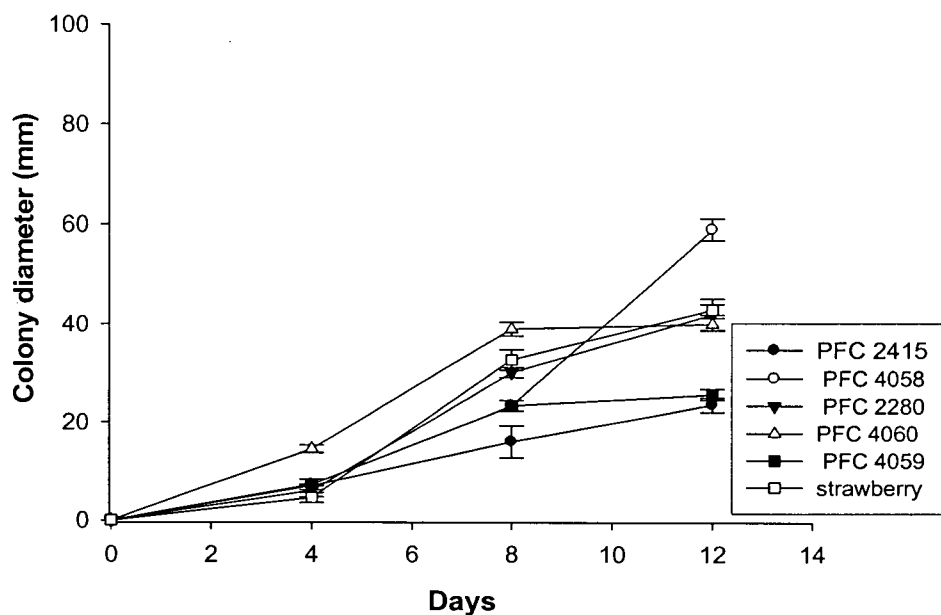
Figure 2-7 Mean colony diameter (mm) for all 6 isolates of *C. gloeosporioides* during the 12-days of incubation at 15°C. I represents the standard error of the mean (S.E.) n=3 (measured from 3 different plates).
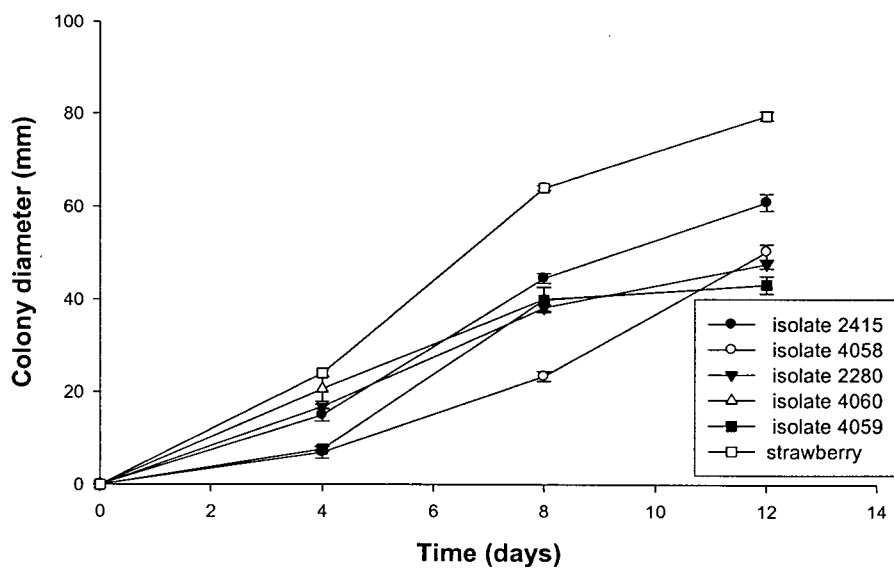
Figure 2-8 Mean colony diameter (mm) for six isolates of *C. gloeosporioides* during 12 days of incubation at 20°C. n=3 (measured from 3 different plates).
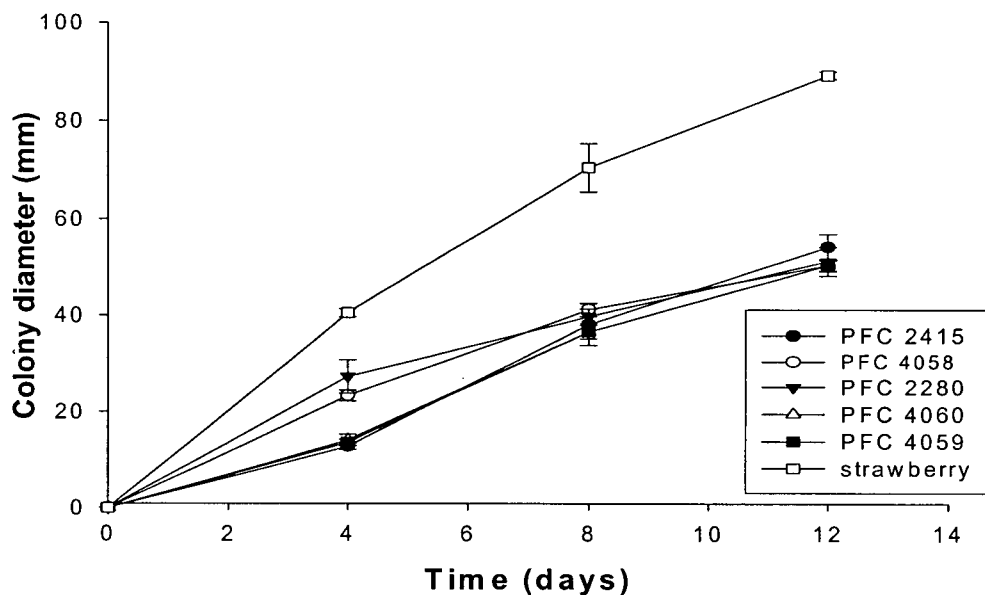
Figure 2-9 Mean colony diameter (mm) for all 6 isolates of *C. gloeosporioides* during the 12-days of incubation at 25°. n=3 (measured from 3 different plates).

2.3.4 Percentage and rate of conidia germination at varying temperatures

2.3.4.1 Percentage of conidia germination for the PFC isolates after 24 hours

Figure 2.10 shows the percentage of conidia germinated after twenty-four hours revealed that the optimum conidia germination temperature was observed between 15 to 30°C. Two-way ANOVA analysis of the conidia germination after 24 hours revealed that significantly lower conidia germination occurred at 4°C and 35 °C. At 10°C, a two-way ranked ANOVA revealed a significant lower conidia germination for PFC 4058 and 4059 (p=0.01) than PFC 2415, 4060, and 2280 after 24 hours. At 15°C, a two-way ranked ANOVA revealed significant higher conidia germination percentage for PFC 4058 and 2415 than PFC isolates 4060, 2280 and 4058 after 24 hours. No differences in conidia germination were detected at 20 and 25°C. At 35°C, a two way ranked ANOVA detected a significant lower conidia germination percentage for PFC 4058 conidia (p=0.01) than

PFC 2415, 4058, 2280 and 4059 after 24 hours. All the isolates performed poorly (low percentage of conidia germinated) incubated at 4 °C and 35°C.
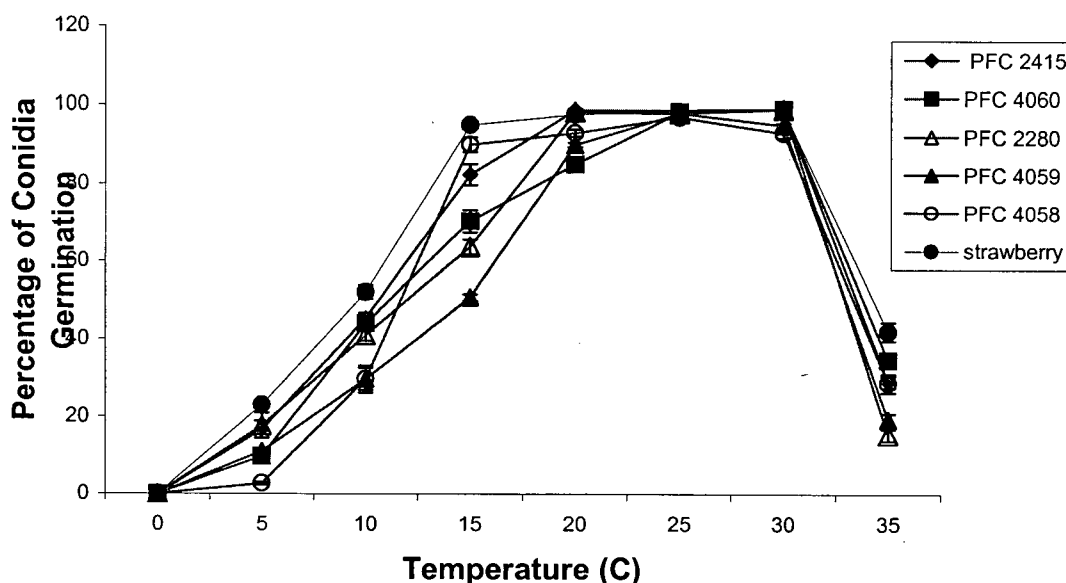
Figure 2-10 Mean percentage of conidia germinated for the six *C. gloeosporioides* isolates after 24 hours for the temperatures of 4-35 °C. Bars measure is the standard error of the mean. n=3 (conidia counted from 3 different plates).

2.3.4.2 Determining the number of hours for 50% conidia germination for each PFC isolate.

Figure 2.11 illustrates that the optimal conidia germination for all the isolates ranged between 20-30°C with conidia germination time ranging from 7-9 hours for the 50 % conidia germination trial. At 4 °C, a two way ranked ANOVA detected faster conidia germination for PFC 4058 (35 hours) compared to PFC 4060 (52 hours) and 4059 (47 hours). No significant differences in conidia germination were noted between the PFC isolates at 10°C. For 15°C, a two-way ranked ANOVA detected no significant differences between PFC isolates although PFC 4058 (17.0 hours) demonstrated the fastest conidia germination rate compared to PFC 2415, 4060, 2280 and 4059 (20-24 hours). No significant differences in the conidia germination were detected between the PFC isolates grown at 20, 30 and 35°C.
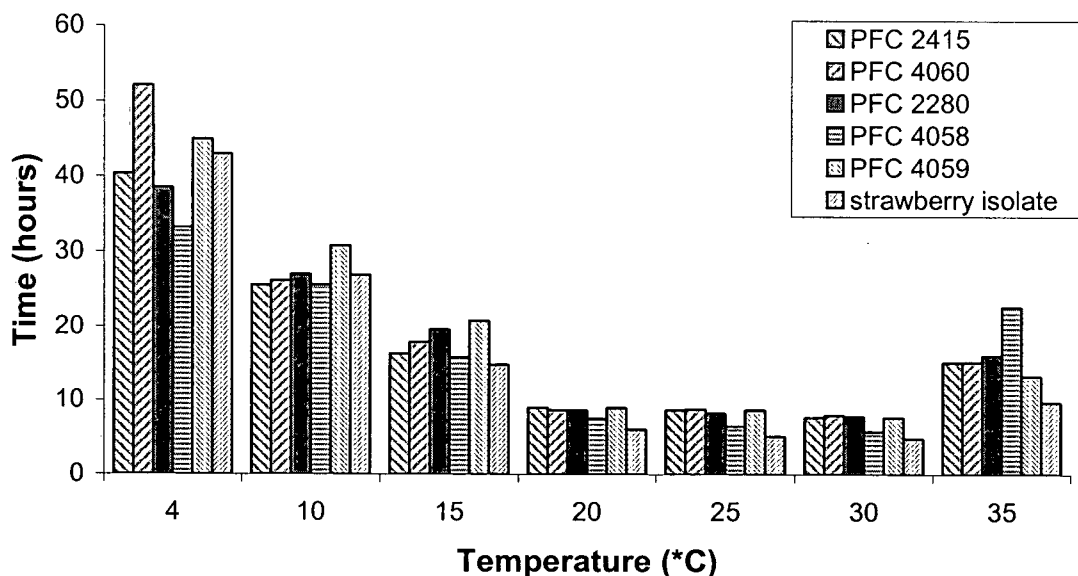
Figure 2-11 Mean number of hours for 50% conidia germination for the six *C. gloeosporioides* isolates at temperatures ranging from 4 to 35°C. n=3

2.3.5 Inoculation of *A. tsugense* in a small trial

Table 2.4 shows the result three weeks after the application of the treatments. One-way ranked ANOVA detected no significant difference in the percentage of disease shoots between the PFC isolates although PFC 4059 showed the highest percentage of diseased shoots. Only one replicate was used for diseased berries therefore no statistical analysis was performed. After 3 weeks, the highest percentage of diseased berries occurred on PFC 4060 compared to the other PFC isolates.

Table 2.5 shows the result two months after the isolates were applied. One-way ranked ANOVA detected no differences in percentage of diseased shoots between all the PFC isolates although PFC 2280 showed the highest percentage of diseased shoots. After 2 months, the highest percentage of diseased berries occurred on PFC 4060 compared to the other PFC isolates. Figure 2.12 and 2.13 show dwarf mistletoe swelling before and 2 months after a spray with isolate PFC 2415.

Table 2-4 Percentage of diseased shoots and berries 3 weeks after treatments were applied at the PFC.

Trt	Isolate description -Stabileze formulation	# of reps	% Shoots diseased	Total # of shoots	% Berries diseased*	Total # of berries *
1	PFC 2415	3	9	154	33	30
2	PFC 2280	3	10	149	20	50
3	PFC 4060	3	5	99	75	20
4	PFC 4059	3	12	82	0	45
5	PFC 4058	3	8	68	20	100
6	Control	3	2	81	0	0

- *One female replicate for treatment. Three swelling were used for each treatment.

Table 2-5 Percentage of diseased shoots after 2 months treatments were applied at the PFC site.

Treatment	Isolate **	Replicates (number of swellings in a treatment)	% of shoots diseased	Total number of shoots	%of berries diseased *	# of berries *
1	PFC 2415-	3	18	154	60	30
2	PFC 2280-	3	22	149	25	50
3	PFC 4060-	3	15	99	75	20
4	PFC 4059-	3	18	82	33	45
5	PFC 4058-	3	19	68	33	100
6	Control	3	2	81	0	0

- *One female replicate for treatment. Three swelling were used for each treatment.

- **Each isolate was formulated with the Stabileze process.

Figure 2-12 Healthy *A. tsugense* berries and shoots before formulated treatment was applied.

Figure 2-13 Healthy and diseased *A. tsugense* berries and shoots, 2 months after treatment 2415 were applied at the PFC trial.

2.3.5.1 Confirmation for the presence of *C. gloeosporioides* on diseased shoots and berries

On October 21, 2002, diseased *A. tsugense* shoots were collected from each of the treatments and placed on PDA, using the sterile technique. Table 2.6 shows the results of *C. gloeosporioides* re isolations from shoots and berries collected at Spider Lake site on October 21, 2002. *C. gloeosporioides* was re isolated from diseased shoots from all the inoculum treatments (33 to 100% re isolation of *C. gloeosporioides*). No *C. gloeosporioides* was re isolated from the control treatment.

Table 2-6 *Colletotrichum gloeosporioides* **(C. g.) reisolations collected on October 21, 2002**

Treatment	Number of replications	Number of shoots per replicate	Shoot and berry health	Percent re-isolated with *C.g.* from diseased shoots (%)*
1	3	3	Diseased shoots	67
2	3	3	Diseased shoots	100
3	3	3	Diseased shoots	33
4	3	3	Diseased shoots and berry	67
5	3	3	Diseased shoots	33
6	3	3	Healthy shoots	0

- ***Successful re-isolation was defined as *C. gloeosporioides* colony forming on the PDA plate.**

The temperatures and relative humidity at the PFC field trial site were monitored for 14 weeks after inoculation (Appendixes I–IV). All 5 PFC isolates and a water control formulated with 'Stabileze' were applied to the tagged dwarf mistletoe swellings on the morning of July 31, 2002. The temperature and relative humidity during the time of application was 24°C and 50 % respectively. On July 31, the day of inoculation of the treatments, the maximum day temperature was 32°C at 12:48 PM while lowest humidity was 20% at 2:48 PM. The lowest temperature within the first 24 hours was 13°C, which occurred at 8:30 AM on August 1, 2002.

After treatments were applied to the tagged dwarf mistletoe swellings, diseased shoots and berries were assessed in the second and third week, followed by monthly assessments that were continued until March 2003. After 3 months, it was impossible to accurately access true shoots because new shoots developed and previously counted diseased shoots disappeared.

2.4   Discussion

The evaluation of *C. gloeosporioides* as a biological control agent in this study focused on the development tests used for this study including (1) determining a medium for mass production of conidia; (2) testing *in vitro* virulence; and (3) determining optimum temperatures for mycelial growth, conidia germination and conidia survival;

and field survival. Knowledge of optimum temperature for mycelial growth rate and conidia germinations temperatures for the biological control agent can help establish the best application climatic conditions for rapid conidia germination and mycelial growth of *C. gloeosporioides* in field conditions (Templeton 1982; Templeton et al. 1986; Watson 1998; Wraight et al. 2001).

To enhance maximum sporulation for a biological control agent, a substrate with a good surface area to volume ratio is essential. Substrate particles (in this case cereal grains) that remain separated after hydration and sterilization increase this ratio, allowing for more space on which the fungus can sporulate (Jenkins et al. 1998). For this study, millet was the easiest to mix and it maintained its structural integrity after autoclaving compared with the rice, wheat bran, and slow oats. Based on comparisons of the mean number of conidia on the different substrates for the 5 PFC isolates, the millet substrate produced significantly more conidia than rice, followed by the wheat bran and slow oats substrates (Figure 2.3). Ramsfield (2002) found that millet was well suited for the production of *C. gloeosporioides* infecting lodgepole pine. PFC 2415 produced the highest number of conidia on the millet media although not significantly different from PFC 4060, PFC 4058, and PFC 2280.

The nutritional environment of the substrate can be important to optimize the fitness of the biological control agent (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson 1997; Jenkens et al. 1998). Fitness is defined as rapid conidia germination, high rates of appressorium formation, and the tolerance of desiccation for effective formulation and long shelf life (Wraight et al. 2001). Researchers working with *C. truncatum* have found that the conidia virulence and production of conidia can be increased by optimizing the ratio of C: N in liquid or solid media. Ratios of 20:1 and 15:1 were more efficacious in inducing disease on *Sebania exaltalta* (Raf.) Rydb. than 30:1 and 10:1 (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson 1997). Other benefits from using 15:1–20:1 included rapid germination of conidia, high yields of conidia, higher number of appressoria formation, and enhanced disease formation on *S. exaltalta* (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson 1997; Wraight et al. 2001). Future work on producing *C. gloeosporioides* conidia may include trials on

C: N ratios in both a liquid and solid media that may increase the virulence and production of conidia for hemlock dwarf mistletoe *C. gloeosporioides* isolates.

In this study, dwarf mistletoe infected hemlock trees grown in pots were not available. Ideally, the biological control agent should be screened on intact plants (*in vivo*) rather than detached plants (*in vitro*) (Faultin et al. 2004). *In vitro* screening of the PFC isolates allowed for a rapid and less labor intensive isolate selection than *in vivo* screening (Faultin et al. 2004). Pathogenicity screening for the 5 isolates was conducted on detached swellings of dwarf mistletoe (Winder and Watson 1994; Oleskevich et al. 1998). A hemlock branch infected with dwarf mistletoe, placed with the proximal end in a saturated hydroponics block, was the most efficient way of ensuring virulence screening. Dwarf mistletoe shoots without the hemlock branch did not remain viable for the duration of the testing period. Alosi and Calvin (1985) and Kuijt (1960) suggested rapid wilting of detached aerial shoots can commonly occur as a result of high transpiration rates and relative low turgour pressures in the dwarf mistletoe aerial shoots. Dwarf mistletoe stems attached to the hemlock branch remained intact when humidity within the germination box was below 80%. High relative humidity (above 80%) within the seed germination box resulted in the dwarf mistletoe shoots breaking into segments. One the most important steps in preparing a virulence test with detached hemlock branch infected with dwarf mistletoe is to remove the excess moisture from the bottom seed germination tray. Deeks et al. (2000; 2001) developed a tissue culture technique using dwarf mistletoe seed and induced callus tissue as a method of studying the relationship between the biological control agent and dwarf mistletoe. Virulence screening of biological control agents with callus tissue may not be realistic, because callus is undifferentiated tissue and does not represent differentiated mistletoe tissue. Detached leaves and stems are often used to determine the virulence of different isolates including *Sclerotina* spp. on Soya bean (Chun et al. 1987; Green 1995; de Rio 2002), *Phoma* on *Rubus* (Sumanpong 2005), and *Fusarium* on *Rubus* (Oleskevich et al. 1998). In this study, PFC 2415 demonstrated the highest *A. tsugense* shoot and berry disease *in vitro* conditions although the results were not significantly different than the other isolates.

The linear growth characteristics of the five PFC isolates were investigated to determine the maximum, minimum, and optimum growth temperatures. Linear growth is

essential for the development of *C. gloeosporioides* as a biological control agent and allows the researcher to screen an isolate based on climatic growth parameters for a particular isolate (Bailey and Jeger 1992; Templeton 1982; Watson 1998). The temperatures resulting in maximum mycelial growth for the PFC isolates ranged between 20 and 25°C with the exception of PFC 4058 (with a growth rate of 4.1 mm/day) (Figures 2.7 and 2.8). Ramsfield (2002) found that the optimum temperature range of *C. gloeosporioides* that infects lodgepole pine was between 15 and 25°C with a growth rate ranging from 3–4 mm/day. Similar growth ranges occurred for the biological control agent *C. gloeosporioides* for *Hypericum perforatum* L. (St John's Wort) and for *C. gloeosporioides* that attacks the *Hakea* spp., with an optimum temperature for mycelial growth for both these *C. gloeosporioides* isolates was between 15 and 25°C (Hildebrand and Jensen 1991; van Rooi and Wood 2003).

PFC 4058 maximum linear growth occurred at 15°C with a 4.1 mm/day growth rate. At 15°C, PFC 4058 requires 16 hours for 50% conidia germination while the other PFC isolates required 18-22 hours. This suggests that PFC 4058 is more adapted for mycelial growth and conidia germination at lower temperatures than the other PFC isolates. PFC 4058 may be better suited for application on cooler days or later in the season. For this research only 3 replicates were used per isolate for determining conidia germination rates and mycelial growth. Further work on isolate PFC 4058 would be required to determine the optimum temperature for conidia germination and mycelial growth.

The percentage of conidia germination and speed of conidia germination are important growth parameters to determine since they give an indication of the environmental requirements and fitness for a particular fungus (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson 1997; Jenkins et al. 1998). Conidia germination can be used to (1) determine the temperature ranges for conidia germination and disease development, and (2) to determine the number of hours for a particular biological control agent to initiate penetration and infection on the host under various temperatures (Templeton 1982; Watson 1998). As the length of time between inoculum application and conidia germination increases, the probability of infection decreases because of abiotic factors such as relative humidity, exposure to rain-wash, abrasion, ultraviolet light damage, and desiccation that reduce the viability of the conidia (Green et al. 1997; Boyetchko et al.

1998). Speed of conidia germination is also important for fungal pathogenicity, since it has direct effect on the formation of appressoria, and ability to infect or penetrate the host plant (Bailey and Jeger 1992; Green et al. 1997; Wraight et al. 2001). For all the isolates, 95-100 % germination occurred after 24 hours for the temperatures between 15-30 °C. The conidia germination at the temperatures 4, 10 and 35°C may hinder the development of the disease on the hemlock dwarf mistletoe. Examples of some of the limiting factors for disease development by *C. gloeosporioides* on other plant species include temperature, duration of wetness or dew period, growth stage, and inoculum concentration for *C. gloeosporioides* (Chongo and Bernier 2000). Similar optimum environmental conditions for field application of *C. gloeosporioides* on hemlock dwarf mistletoe for infection and disease development should be determined to enhance the efficacy of this biological control agent (Templeton 1982; Watson 1998). Appressoria can remain dormant in unfavourable conditions until the environment becomes favourable. Slowing of the appressorium development may reduce the efficacy of the fungus for successful infection (TeBeest et al. 1978).

The 50% conidia germination test detected that the optimum conidia germination rate occurred for the temperatures between 20 to 30°C (7.8–9.2 hours) for all the isolates. At 15, 10, 20, 25, 30 and 35°C, no significant differences were detected between all the isolates. At 15°C, 4058 showed a faster germination rates although not significantly different than the other PFC isolates. PFC 4058 showed slightly faster conidia germination rate compared to the other PFC isolates at 20, 25, and 30°C. At 35°C, PFC 4058 demonstrated the slowest conidia germination time. Future pathogenicity studies under field or greenhouse conditions may determine conidia germination rates and efficacy of PFC 4058 in an *in vivo* situation.

One of the limitations of using biological control agents in the field is the length of dew period. The PFC isolates required 9 hours or longer before infection could occur on *A. tsugense*. To overcome the dew period at the outdoor PFC site, the PFC isolates were formulated with 'Stabileze method' (Green et al. 1997; Boyetchko 1998; Quimby et al. 1999).

During the 1-year assessment period, no differences in the number of diseased shoots were detected between PFC isolates at the Pacific Forestry Centre field site. Two months

after the application of the PFC isolates, a relatively low percentage of diseased dwarf mistletoe shoots were noted in all the treatments. Although, the PFC isolates (or treatments) showed higher percentage shoot disease than the control treatment. Low infection rates and disease developed in the shoot treatments may have resulted for the following reasons:

1. The dwarf mistletoe plants at the PFC site were mature, hardened off plants. Chongo and Bernier (2000) detected that *C. gloeosporioides* was effective in controlling younger lentil seedlings than hardened off older lentil plants. Similar trends in the reduction of dwarf mistletoe plants may occur with PFC isolates (Chongo and Bernier 2000);

2. The concentration of conidia may have not favored disease infection (Chongo and Bernier 2000);

3. The PFC isolates may be more effective on dwarf mistletoe berries than dwarf mistletoe shoots.

In vitro studies may not represent the *in vivo* situation due to the fact that *in vitro* may not take into consideration factors such as fluctuating temperature and humidities and antagonistic microorganisms that can reduce the efficacy of the biological control agent (Wraight et al. 2001). Environmental conditions such as high or low temperatures, poor spore dispersal, ultraviolet light, low humidity, and the drying effects of the wind may have prevented (killed the conidia) or decreased the viability of conidia resulting in low infection rate by the biological control agent (Green et al. 1997; Boyetchko et al. 1998).

Factors contributing to the underestimating the disease incidence on the dwarf mistletoe shoots may have included the accuracy of the disease shoot counts. Inaccuracies in determining the percentage of diseased dwarf mistletoe shoots may have arised from;

1. New buds on the tagged dwarf mistletoe plants developing into shoots after the initial assessment and/or

2. Diseased shoots that dropped between assessments which underestimated the infection assessment.

Differences in percentage of diseased shoots between PFC isolates at the PFC site trial were not significantly different. Factors that may have resulted in accepting the null

hypothesis and concluding that no differences in the percentage of diseased shoots and berries between the PFC isolates at PFC site may include;

1. The low number of replicates per treatment and/or,

2. A high variation in the number of diseased shoots within and between treatments.

Two months after isolates were applied to the dwarf mistletoe swellings, the percentage of diseased dwarf mistletoe berries ranged from 33-75% for the field trial with PFC 4060 showing the highest percentage of diseased berries. Unfortunately there was only one female swelling per treatment due to lack of female experimental units. Shoot reductions for the field study ranged from 15-22% after 2 months with PFC 2280 showing the highest percentage of diseased shoots. *In vitro* studies showed that the disease incidence as result of applying the PFC isolates on dwarf mistletoe swellings ranged from 30-60% on dwarf mistletoe shoots and 50-85 % on dwarf mistletoe berries. With this study, it appears that *in vivo,* PFC isolates appear to attack the dwarf mistletoe berries more successfully than the dwarf mistletoe shoots. Further work is required to verify this conclusion.

2.5   Conclusion

Ideally, selection of an inundative biological control agent is based on the most aggressive and virulent isolate and its ability to survive in field conditions with relatively no impact on non-target plants (Templeton 1982; Watson 1998). A major objective of the studies described in this chapter was to select a single isolate for further extensive field testing. Obviously the isolate to be selected must produce conidia readily. The differences in conidia production, however, were so small that none of the isolates could be rejected on that basis. The most important criterion for selection is undoubtedly virulence. In this chapter virulence was tested directly, both *in vivo* and *in vitro*. It was also tested indirectly as rate of germination (quick germination presumably being associated with greater virulence) and linear mycelial growth rate (again greater rates of growth presumably being associated with greater virulence). The direct tests of virulence suggested variation between isolates although statistical tests failed to prove this conclusively. Nevertheless PFC 2415 is most likely to be the most virulent of the isolates. In the case of germination rate and linear growth rate there was an interaction between isolate and temperature – the best isolate depended on the temperature at which the test

was done. This suggests that eventually different isolates might be used for different locations. The selection of an isolate was based on prioritizing the screening results for each PFC isolate tested. For the purpose of further study (described in the next chapter) the results of the direct virulence tests were judged to outweigh other considerations, and so isolate PFC 2415 was selected as the lead isolate.

3 CHAPTER 3- SPIDER LAKE TRIAL

3.1 Introduction

An inundative biological control strategy involves the use of an indigenous fungus that is applied in massive dosages to control or suppress the target organism. The use of an inundative biological agent may provide an alternative method to traditional silvicultural practices used to reduce hemlock dwarf mistletoe in variable retention harvest systems, especially in riparian and other ecologically sensitive areas (Muir 2003). In this study, the native fungus *C. gloeosporioides* was assessed as a candidate for inundative biological control of western hemlock dwarf mistletoe.

The application of a biological control agent in the field requires a thorough understanding of its biology. For example, growth parameters can vary with different fungal genera, and even with different isolates within the same fungal species (Wraight et al. 2001). To improve the potential of a biological control agent in field conditions it is important to determine 1) temperature ranges for the biological control agent infection that are conducive in the host environment, 2) an efficacy isolate, and 3) production and formulation methods that increase the potential for infection by the biological control agent (Jenkins et al. 1998; Wraight et al. 2001). Ideally, an effective isolate will quickly suppress its host or attack a particular host stage, through a one time application of an isolate, leading to an economical feasible reduction or a long-term suppression of the host (Wraight et al. 2001 Sheppard et al. 2003).

Most biological control agents require exact moisture and temperature conditions for germination and host penetration. These conditions may not frequently occur in the field and their absence will result in ineffective biological control (TeBeest 1985). For many foliar pathogens, spore adhesion to the phylloplane and extended dew periods are required for spore germination (Hoagland 1990). Other factors that can reduce spore germination are temperature extremes, ultraviolet radiation, desiccation, and humidity fluctuations (Green et al. 1997; Boyetchko et al. 1998). Many biological control agents require a 6-24 hour period of moist conditions (dew period) for the germination of the propagules and penetration into the host (Green et al. 1997; Boyetchko et al. 1998). To overcome these environmental constraints the biological control agent is incorporated

into a formulation (Boyette 1994; Green et al. 1998; Boyetchko et al. 1998; Wraight et al. 2001).

A bioherbicide formulation is defined as a combination of an active ingredient (biological control agent) and additives in a single package that possesses desirable physical and chemical properties to control undesirable plants, insects and diseases (Green et al. 1997; Boyetchko et al. 1998; Wraight et al. 2001). Formulations enable the spread of a very small amount of microbial propagules to a target plant over a wide area, enhance shelf life, and increase efficacy without adversely affecting the effectiveness of the agent (Wraight et al 2001; Sheppard et al. 2003).

Liquid and solid based (called a wettable power) formulations were used in this study. In liquid formulations adjuvant compounds are used in a solution that assists or modifies the action of the principle active ingredient (Green et al. 1997; Boyetchko et al. 1998). Adjuvants can also improve or modify germination, increase virulence and assist in biological control agent delivery. Adjuvants include a range of compounds such as surfactants, stickers, inert carriers, anti-evaporation agents and micronutrients (Green et al. 1997; Boyetchko et al. 1998). Compounds such as paraffinic, mineral and soybean oils, corn syrup and sucrose can be used as adjuvants (Jackson 1997; Green et al. 1997; Boyetchko et al. 1998). An adjuvant is any substance that can enhance the efficiency of a biological control agent (Green et al. 1997). For example, the adjuvant sucrose added to the fungus *Alternaria macrospora* and sprayed on *Anoda cristata* (Spurred Anoda) resulted in an increase in disease severity (Green et al 1997; Shabana et al. 2001). Once a liquid formulation is made up it has viability of 2-4 days (Jackson and Bothast 1990). Jackson and Bothast (1990); Schisler et al. (1996) found that a sucrose and gelatin formulation increased the efficacy of *Colletotrichum truncatum* sprayed on *Sida exaltata*. The sucrose and gelatin formulation generally resulted in a higher number of conidia sticking to plant surfaces than conidia suspended in water (Trujillo et al.1994; Schisler 1996; Green et al. 1997; Boyetchko et al. 1998). Sucrose also provides nutrition to the biological control agent (Green et al. 1997; Boyetchko et al. 1998). For the field trial described for in this chapter, a liquid formulation, containing a mixture of 2% sucrose and 0.5% gelatin (Trujillo et al. 1994: Trujillo et al. 2001) was used.

Wettable powders are also used as foliar sprays. Solid formulations utilize grains, peat, clay, wheat-gluten matrix, vermiculite or starches as carriers. Carriers should be inexpensive, easily sterilized, non-toxic and consistent in physical properties. Carriers should also provide nutrition, act as a buffer to environmental extremes, prevent rain wash-off and protect the propagules from dehydration (Green et. al. 1997; Boyetchko et al. 1998). Wettable powders can be stored for long-term use. Most liquid formulations have shorter shelf life than wettable powders (Green et al. 1997).

A wettable powder formulation involves harvesting spores and mixing them with an inert carrier. This process results in a dry powder that can be stored and resuspended in water. Fungal spores must be able to withstand the process of drying, storage and dehydration without an appreciable loss of viability. An example of a wettable powder is a formulation called 'Stabileze.' The'Stabileze' process consists of adding the concentrated fungal propagules (biological control agent) suspension to water absorbent starch sucrose and corn oil. Silica is mixed into the matrix and a granular product is formed (Quimby et. al 1999; Zidack and Quimby 2000). 'Stabileze' was first developed for *C. gloeosporioides* and *Fusarium oxysporium*, and it was selected as one of the formulations for the current field trial.

The ultimate goal of this project was to develop a method that can substantially reduce the spread of hemlock dwarf mistletoe by reducing the ability of this parasitic plant to produce seeds. A biological control agent that can kill the endophytic system of hemlock dwarf mistletoe would have the greatest effect on reducing dwarf mistletoe spread, intensification, and damage (Shamoun et al 2004). Parmeter and others (1959) observed *C. gloeosporioides* invading the endophytic system of *A. abietinum*. Ramsfield (2002) did not detect the presence of the biological control agent *C. gloeosporioides* in the endophytic system of *A. americanum*. *C. gloeosporioides* is usually parasitic on shoots, pistillate flowers and fruits of dwarf mistletoes (Muir 1967; Kope 1997: Kope and Shamoun 2000). Short-term control of dwarf mistletoe can be achieved when the biological control agent destroys the current aerial shoots and berries (Shamoun and DeWald 2002; Shamoun et al. 2003).

The objectives of the field study at Spider Lake were:

1. To determine the degree of control that can be achieved with *C. gloeosporioides* in a field situation;

2. To compare two formulations;

3. To determine the persistence of *C. gloeosporioides* after the application of the treatments;

4. To determine whether *C. gloeosporioides* can invade the endophytic system of *A. tsugense.*

3.2   Methods and Materials

3.2.1   Stabileze and sucrose and gelatin formulations

The two formulations used at the Spider Lake trial were 'Stabileze' (Quimby et al. 1999) and a 2% sucrose and 0.5 % gelatin formulation. Preparation of conidia prior to formulation included the same procedure outlined in 2.2.1.1 in Chapter 2. For the Spider Lake trial the lead isolate PFC 2415 was cultured on PDA (Difco). PFC 2415 was originally isolated from diseased berries of western hemlock dwarf mistletoe from the District of Horne Lake near Parksville, Vancouver Island. Conidia production was quantified for each flask by using a haemacytometer to count the number of spores with a compound microscope using in a 10^{-2} dilution of the stock suspension. The stock suspension of *C. gloeosporioides* was then adjusted to a concentration of 10^7 conidia/mL for the 'Stabileze' and 1.0×10^6 conidia/mL for the sucrose gelatin formulation.

The procedure used in Spider Lake field trial for making the 'Stabileze' and 2% sucrose and 5% gelatin formulations is outlined in Chapter 2.

3.2.2   Testing the viability of 'Stabileze' formulation and sucrose gelatin formulation

Both the 'Stabileze' and sucrose/gelatin inoculum formulations were tested for viability before and after the formulations were applied in the field. Each formulation was sprayed onto three PDA plates to determine the presence of *C. gloeosporioides.*

3.2.3   Spider Lake site description.

A field site with western hemlock trees infected with dwarf mistletoe was selected at Horne Lake in the Nanaimo Forest District on Vancouver Island. The site was located west of Qualicum Beach on Horne Lake road on Block # 347, Plan # 33670, in District #3. The GPS reading for the site was 49' 21' 43" N 124' 37' 42" W at 51 meters elevation. The field site was located in the Coastal Douglas-fir Zone (Meidinger et al. 1991) and approximately 2 ha in size. The stand consisted of a young, naturally regenerated, hemlock stand heavily infected by dwarf mistletoe. The majority of the infections were found between 1 and 2 m of the ground.

Both *C. gloeosporioides* and *N. neomacrospora* were found on *A. tsugense* shoots in and around the research site. Before treatments were applied samples with symptoms of *C. gloeosporioides* infected shoots and *N. neomacrospora* hemlock branches were brought back to the lab and identified by aseptic culturing. Cultures were identified by morphological characteristics using a compound microscope.

3.2.4   Treatment and experimental design

The experimental design was a complete random design. A total of 240 clean (showing no evidence of fungal parasites) individual *A. tsugense* infections were assigned to 7 treatments Table 3.1 summarizes treatments, treatment description and the number of experimental used for each treatment for the Spider Lake site

A Hobo™ data logger (Onset, Bourne, MA, USA) was installed near the centre of the plot to record temperature and the relative humidity (Appendix VI-X).

To compare the efficacy of *C. gloeosporioides* in the field, both 'Stabileze' formulated with *C. gloeosporioides* and a *C. gloeosporioides* conidia suspension in 2 % sucrose and 0.5 % solution was used. Three treatments with conidia suspended in a formulation were sprayed on *A. tsugense* swellings. The respective controls were formulated and suspended in sterilized water and sprayed on treatments 4, 5 and 6. Treatment 7 was a sterilized distilled water spray. The treatments included spraying 'Stabileze' formulated with PFC 2415 onto cut and uncut *A. tsugense* shoots. The 2 % sucrose and 0.5% gelatin formulated with PFC 2415 were sprayed onto uncut *A. tsugense* shoots.

The dwarf mistletoe swellings were sprayed until run off. After the application of treatments, PDA plates were sprayed with each of the treatments to verify the viability of the *C. gloeosporioides.*

Table 3-1 Treatments, treatment description and the number of experimental units used for each treatment for the Spider Lake field site.

Treatment #	Treatment description	# of replications
PFC 2415 formulation treatments		
Stabileze treatment Treatment 1	'Stabileze' suspension	36
Cut Stabileze treatment Treatment 2	Cut shoots treated with 'Stabileze' formulated	35
Sucrose Gelatin treatment Treatment 3	Two percent sucrose and 0.5% gelatin	36
Treatments without PFC 2415		
Stabileze control Treatment 4	'Stabileze' formulated with water	35
Cut Stabileze control Treatment 5	Cut shoots with the 'Stabileze' control formulation	36
Sucrose and gelatin control Treatment 6	Sucrose and gelatin with water suspension	35
Water spray Treatment 7	Water spray (no formulation)	27

Treatments 1, 3, 4, 6, and 7 were assessed for shoot and berry health.

3.2.4.1 Pre-treatment and final assessment data.

Initial and final assessments were recorded for every *A. tsugense* infection including: swelling diameter, swelling length, distance between proximal and distal shoots, number of shoots, number of buds (shoots less than 0.5 cm in height), number of fruit, maximum shoot length, and shoot sex.

3.2.4.2 Post- treatment assessment data.

Assessments of dwarf mistletoe swellings were conducted at 15, 28, 63, 102, 145 and 240 days after treatments were applied. At each assessment date the following data were recorded for each of the treated *A. tsugense* infections; swelling diameter, number of buds, number of shoots, number of diseased shoots, number of berries and the number of diseased berries. Pictures of five swellings per treatment were recorded at each pre- and post assessment date.

3.2.4.3 Treatment application date.

Application of the treatments occurred on August 29, 2002, from 11:30 AM in the morning to 6:00 PM in the afternoon. The weather during the inoculation was sunny with periods of overcast and a maximum temperature of 23.6 °C coinciding with the minimum humidity 52.8% at 3:35 PM. The weather during the following week remained around 20°C throughout the day and night temperatures were as low as 7.0°C (Appendix VI-X).

3.2.5 Culturing from asymptomatic and symptomatic *A. tsugense* shoots

Three months (November 12, 2002), after inoculation, samples of diseased shoots and berries were selected to characterize the typical symptoms of *C. gloeosporioides*. Typical diseased symptoms of *C. gloeosporioides* on *A. tsugense* included small necrotic brown or black lesions that progress from the tip of the shoot to the base of the shoot. Initial disease symptoms were typically brown to black lesions on the tip of the stem. The disease progressed on the dwarf mistletoe shoot by necrosis to complete blackening of shoot. Acervuli were observed mostly on dwarf mistletoe berries in the field at this time. A total of thirty typical samples were selected at this time for positive identification of *C. gloeosporioides*.

The samples were collected and returned to the laboratory where each sample was examined using a dissecting microscope. The dwarf mistletoe shoots were cultured using two different techniques. The first included placing the diseased shoots on moist filter paper and allowing the fruiting structures to emerge. The second method included surface sterilizing the diseased dwarf mistletoe shoots and plating them on PDA. *C. gloeosporioides* identification was based on the presence of acervuli and asexual structure morphology as described by Kope et al. (1997) and Sutton (1980).

Ten months after the inoculation, samples of 10 randomly selected replicates were collected from treatments 1, 2, 4, 6, and 7. The samples were collected and returned to the laboratory where the dwarf mistletoe shoots were examined for any signs of disease using a dissecting microscope. Non-symptomatic and symptomatic *C. gloeosporioides* shoots were collected from each selected swelling. Dwarf mistletoe shoots were then surface sterilized and placed on PDA. The cultures from the dwarf mistletoe shoots were grown for ten days and examined by microscopic techniques. Asexual fruiting structures such as

the conidia and conidiophores were examined and compared to the structures described by Sutton 1980 for *C. gloeosporioides*.

3.2.6 Destructive sampling of hemlock tissue infected with *A. tsugense*.

Treatments 3 and 5 (cut shoot treatments) and 1, 3, 4, 6 and 7 (intact shoots) were sampled to determine if *C. gloeosporioides* penetrates the rhytidome, living bark and wood (living tissue) and dead wood. The rhytidome is defined as the dead bark; living tissue is defined as the periderm and cork cambium, phloem, the vascular cambium, and xylem (water and mineral conducting xylem cells or sapwood) and the wood defined as non conducting xylem or heart wood found in the hemlock branch tissue infected with dwarf mistletoe (Esau 1977). Reisolation of *C. gloeosporioides* in the living tissue and wood could imply that *C. gloeosporioides* can invade the endophytic system of *A. tsugense*.

For destructive sampling, 7-25 dwarf mistletoe infections were collected from each treatment on June 17, 2003 from the Spider Lake site. Table 3.2 outlines the number of experimental units collected for destructive sampling 10 months after treatments were applied. The dwarf mistletoe swellings were placed in a paper bag and labeled. Larger diameter *A. tsugense* infected branches were cut with a band saw into 1 cm pieces. A small piece of tissue (approximately 2 x 2 mm) was sectioned from *T. heterophylla* branch infected with dwarf mistletoe from the rhytidome including around the basal cup, from the living tissue, and the wood. Samples were surface sterilized for 2 minutes in 95 % ETOH, followed by 2-minute wash in 10% bleach and three 2-minute washes in sterilized distilled water. Each sample of the *T. heterophylla* tissue was surface sterilized and placed on a PDA plate. The swelling tag number and location of the tissue was recorded for each sample. For the wood samples, wood tissue was removed using a sterilized scalpel and placed aseptically on a PDA plate.

Table 3-2 Number of experimental units collected for destructive sampling 10 months (June 17, 2003) after treatments were applied.

Treatment	1	2	3	4	5	6	7
Collected	9	22	7	9	14	7	13
Remaining	27	13	29	27	22	28	14
Total	36	35	36	35	36	35	27

3.2.7 Statistical analysis.

All statistical analysis was performed using Sigma Stat 2.03 (SPSS Inc, Chicago, IL) and the graphs were drawn using Sigma Plot version 7.0 (Jandel Corporation, San Rafael, CA). Two-way ANOVA analysis was performed on the *A. tsugense* diseased berry (factors included formulation type and with and without inoculum). In most situations the data did not meet the assumptions of ANOVA and data was transformed using the equation Arc sin root x. Transforming the data corrects non-normality and unequal variances by transforming all the data values. Tukey's paired analysis was used to compare differences between treatments.

Analysis for *A. tsugense* shoot data required the use of repeated measures ANOVA test since the same shoots were assessed over the 240 day assessment period. The rational of using a repeated ANOVA was due to fact that the samples are dependent. This means the standard error of the mean will be smaller than independent samples due to the positive correlation brought about by the relationship within the experimental units.

For the destructive *T. heterophylla* samples, Chi square analysis was used for data with 2 or more treatment comparisons. A Fisher Exact Test was used for comparisons with two treatments. Chi-square and Fisher exact test were calculated by using a contingency table to test the discrepancies between observed and expected frequencies (X^2 test).

The proportion of diseased berries was calculated by dividing the number of diseased berries counted on an assessment period (after treatments were applied) into the total number of berries counted on September 5, 2002 (initial assessment). This approach assumes that all the diseased berries remain attached to shoots. For each experimental unit, the percentage of diseased berries was determined and averaged for each treatment within an assessment. To determine the percentage of diseased shoots within each treatment within an assessment period, the total number of diseased shoots within each treatment for a particular assessment date (after treatments were applied) was divided into the total healthy shoots on August 29, 2002 (initial assessment for shoots) multiplied by 100. The success of reisolation from various tissues including shoots and berries was expressed as a percentage of the number of attempts.

3.3   Results

3.3.1   Stabileze and sucrose and gelatin formulation

3.3.1.1   Testing the presence of *C. gloeosporioides* for the Stabileze formulation and sucrose and gelatin formulation

One week after the incubation of each treatment spray onto the PDA plates the inoculum treatments formed numerous *C. gloeosporioides* colonies. The control treatments (formulated without inoculum) formed no *C. gloeosporioides* colonies. Conidia identification was based on the conidia produced from the fungal colonies formed on the PDA plates. The conidia and other morphological structure that formed on the PDA media from the inoculum ('Stabileze', and sucrose gelatin) treatments were identified with a compound microscope (Kope et al. (1997); Sutton (1992, 1980). Conidia were embedded in the formulation and therefore not visible for conducting germination tests. The exact viability of the sprayed conidia was difficult to determine although abundant *C. gloeosporioides* colonies were observed on the PDA media.

3.3.1.2   Symptoms and signs of *C. gloeosporioides* in the field.

Two months after treatments were applied symptoms of *C. gloeosporioides* on *A. tsugense* shoots collected from the Spider Lake site appeared as brown to black necrotic regions. Mature infections enlarged and girdled the dwarf mistletoe shoot, resulting in death of the distal portion of the dwarf mistletoe shoot. Some dwarf mistletoe shoots turned black and the entire shoot was killed. *A. tsugense* berries initially turn from green-blue to purple black; eventually *C. gloeosporioides* mummified the berry. Table 3.3 describes the reisolation frequency from diseased shoots and berries, not the symptoms described in this paragraph.

Table 3-3 Frequency of isolation of *C. gloeosporioides* from symptomatic shoots and berries two month after treatment.

Diseased dwarf mistletoe structure	Sample size**	# of Plates with C.g recovered*	Percent Recovery
Shoots	24	20	83.0
Berries	12	10	83.0

- ***Seven of the symptomatic berries sampled were from symptomatic shoots that were also sampled.**
- ****Samples from treatment 1, 3, 4, 6, and 7**

3.3.2 Survival of *A. tsugense* swellings at the Spider Lake site.

Table 3.4 summarizes the number and sex of *A. tsugense* experimental units at treatment (August 2002) and 10 months later (June 17, 2003). Ten months after the application of treatments, several replicates were missing in some of the treatments. Reasons for a loss of treatment replicates included self-pruning of hemlock tree, and wind damage to hemlock branches. At the Spider Lake site, some squirrel damage to swellings did occur but no squirrel damage occurred on the experimental units that were tagged for treatments.

For the diseased *A. tsugense* berry analysis the number of experimental units was lower than in the shoots assessment since male plants do not produce berries. Treatments 2 and 5 were not used for *A. tsugense* berry and shoot analysis given that these treatments had cut shoots.

Table 3-4 Number and sex of *A. tsugense* experimental units at treatment (August 29, 2002) and 10 months later (June 17, 2003).

Treatments	Number of experimental units before treatments	Number of female swellings	Number of male swellings	Number of lost experimental units	Number of experimental units after 10 months
Stabileze	36	14	22	0	36
Cut Stabileze	35	17	18	2	33
Sucrose gelatin	36	14	22.	0	36
Stabileze control	35	13	22	0	35
Cut Stabilize control	36	20	16	0	36
Sucrose/ Gelatin control	35	18	17	2	33
Water spray	27	5	22	4	23

3.3.3   The assessment of *C. gloeosporioides* on *A. tsugense* fruit

The cumulative total of *A. tsugense* healthy and diseased berries is shown in Figures 3.1-3.5 for each treatment. The water spray control treatment (Figure 3.1) demonstrated that 26% of healthy *A. tsugense* berries were dispersed before the September 27 date (28 days after treatments were applied) and remaining (all) healthy berries were mainly dispersed from September 27 to November 5 (63 days after the treatments were applied). A low number (8% of the initial berry count) of *C. gloeosporioides* infected berries remained on the water control after November 5, 2002 (63 days after treatments were applied). On November 5, 2002, all healthy *A. tsugense* berries were dispersed and less than 10% diseased berries remained attached to inoculum treated swellings.

The mean percentage of diseased dwarf mistletoe berries was difficult to determine. Dispersal of healthy berries started shortly after treatments were applied, and at the same time, diseased berries may also been shed. The decline in total berries between any two successive observations was therefore partly due to the normal seed release of healthy berries, and partly due to shedding of diseased berries. Similarly, most of the diseased

berries present at any one observation time were probably counted as diseased berries at the previous observation, but some berries may have become diseased since the previous observation, and some berries counted at the last observation may have been shed. The assumption used to calculate the percentage of diseased berries was based on the observation that diseased berries appeared to remain attached to the dwarf mistletoe shoot until they become mummified. Once the dwarf mistletoe berries becomes mummified they are quickly shed. The highest of the count of diseased berries was based on the November 5 date. Figures 3.2 to 3.4 show the number of diseased berries was consistently the highest on that date (63 days after the application of the treatments). The mean percentage of diseased berries was then calculated as that number divided by the total berry count at the time of treatment. Table 3.5 summarizes the number of healthy female swellings and total number of healthy berries per treatment counted on September 5, 2002.

Figure 3-8 shows the mean percentage of diseased berries on September 27 and November 27 2001 as a percentage of total berries at the time of treatment. Transformed (sin arc root x) two-way analysis of variance was used for the September 27 (day 28) and November 5 date (day 102) revealed a significantly higher mean percentage of diseased berries for inoculum treatments ($p = 0.021$ and $p=0.005$ respectively) compared to the control treatments (Figure 3.8). Statistical analysis detected no significant differences in the percentage of diseased berries between the 'Stabileze' and sucrose and gelatin formulation treatments ($p = 0.921$).

Figure 3.6 and 3.7are photographs of a swelling before sucrose gelatin formulation was applied and 2 months after sucrose and gelatin formation was applied.

Table 3-5 The number of healthy female swellings and berries counted on September 5, 2002

Treatments	Number of female swellings	Total number of healthy berries per treatment counted on September 5, 2002*
Stabileze treatment	14	794
Sucrose and gelatin treatment	14	773
Stabilize control	13	283
Sucrose gelatin treatment	18	1120
Water spray	4	370
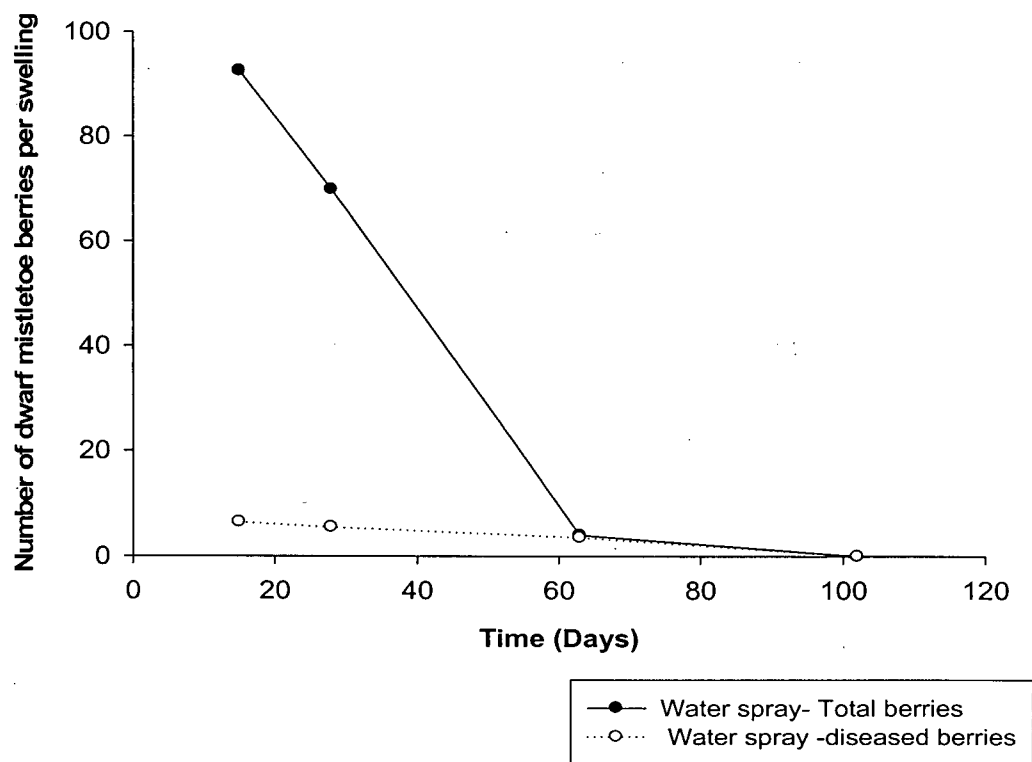
Figure 3-1 Water control-The mean number of total berries and diseased dwarf mistletoe berries from September 12 (day 15) to December 18 (day 102).
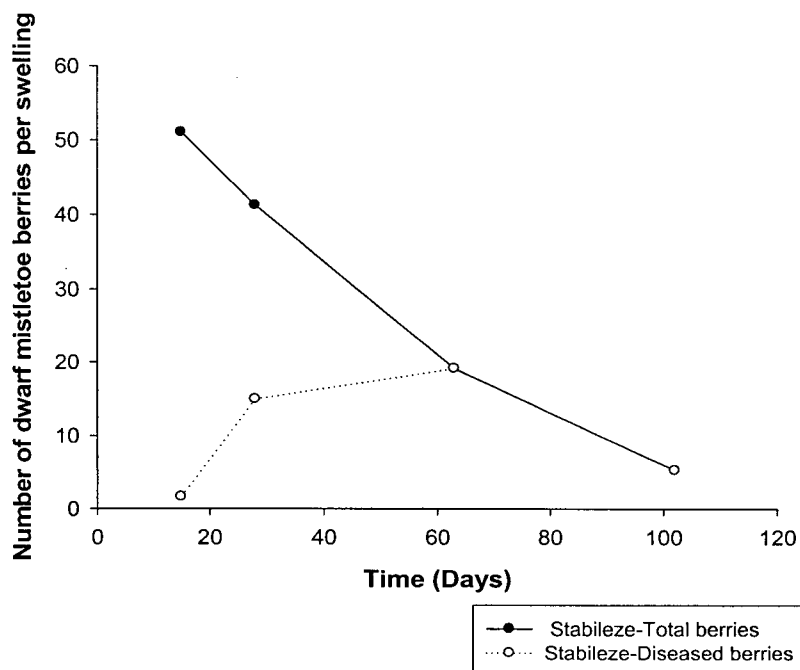
Figure 3-2 Stabileze treatment-The mean number of total and diseased dwarf mistletoe berries from September 12 (day 15) to December 18 (day 102).
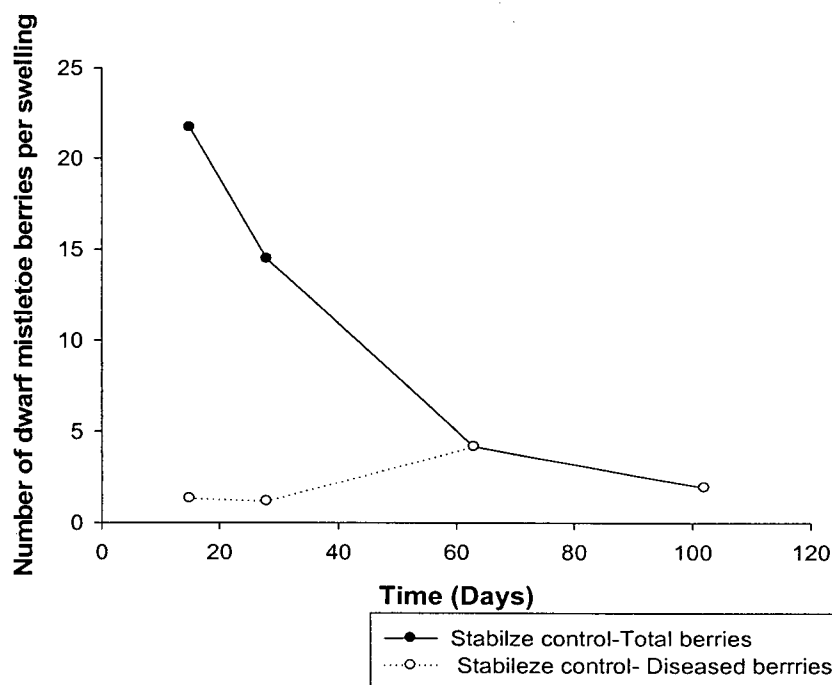
Figure 3-3 Stabileze control-The mean number of total and diseased dwarf mistletoe berries from September 12 (day 15) to December 18 (day 102).
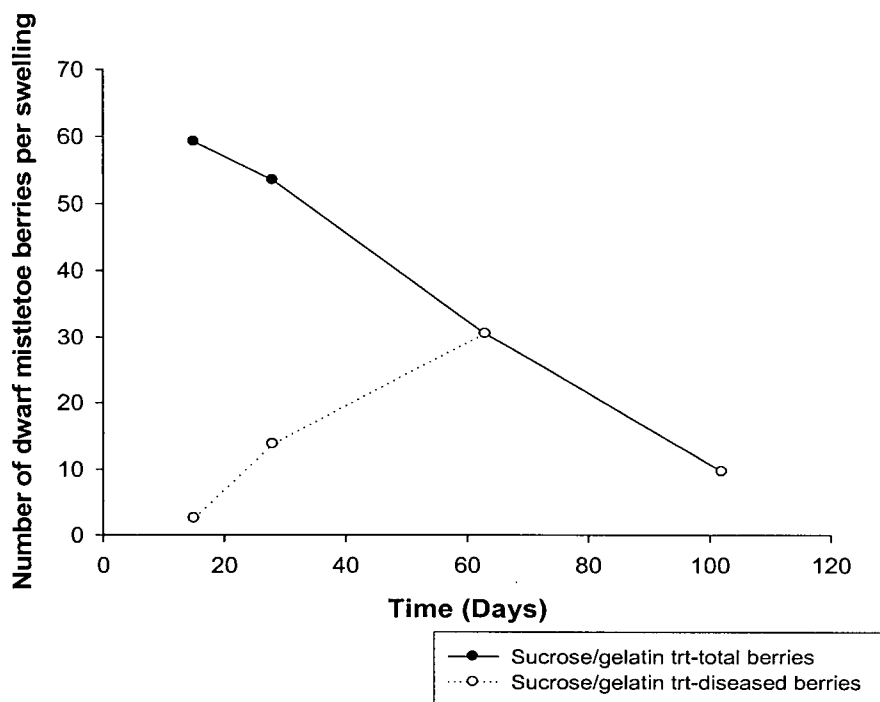
Figure 3-4 Sucrose and gelatin treatment-The mean number of total and diseased dwarf mistletoe berries from September 12 (day 15) to December 18 (day 102).
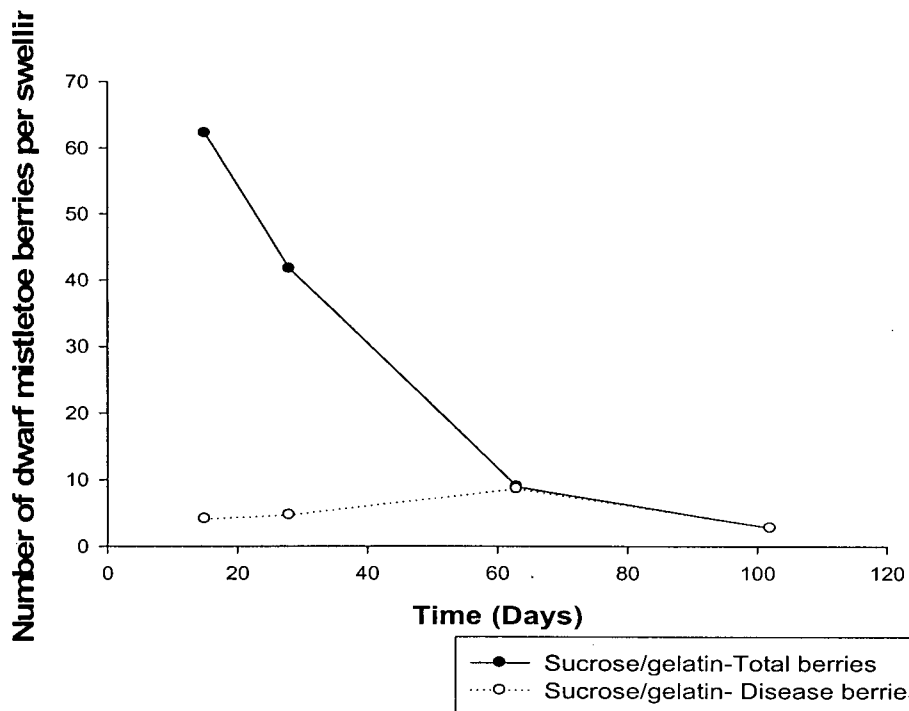
Figure 3-5 Sucrose and gelatin control-The mean number of total and diseased dwarf mistletoe berries from September 12 (day 15) to December 18 (day 102).
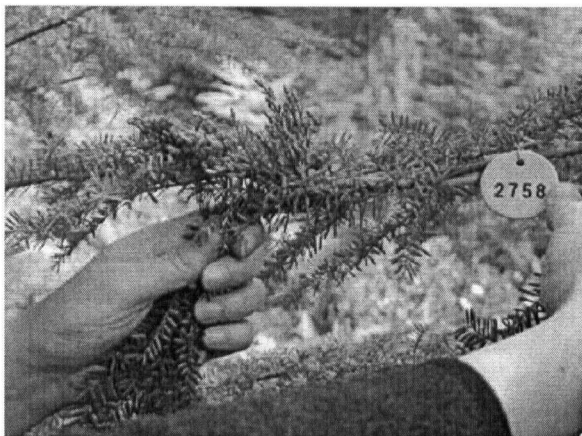
Figure 3-6 Sucrose and gelatin treatment formulated with PFC 2415. Picture of swelling 2758 on August 29, 2002(before treatments were applied)

Figure 3-7 Sucrose and gelatin treatment formulated with PFC 2415. Picture of swelling 2758 on November 5, 2002 (63 days after the treatment was applied). Note the blackened diseased berries.
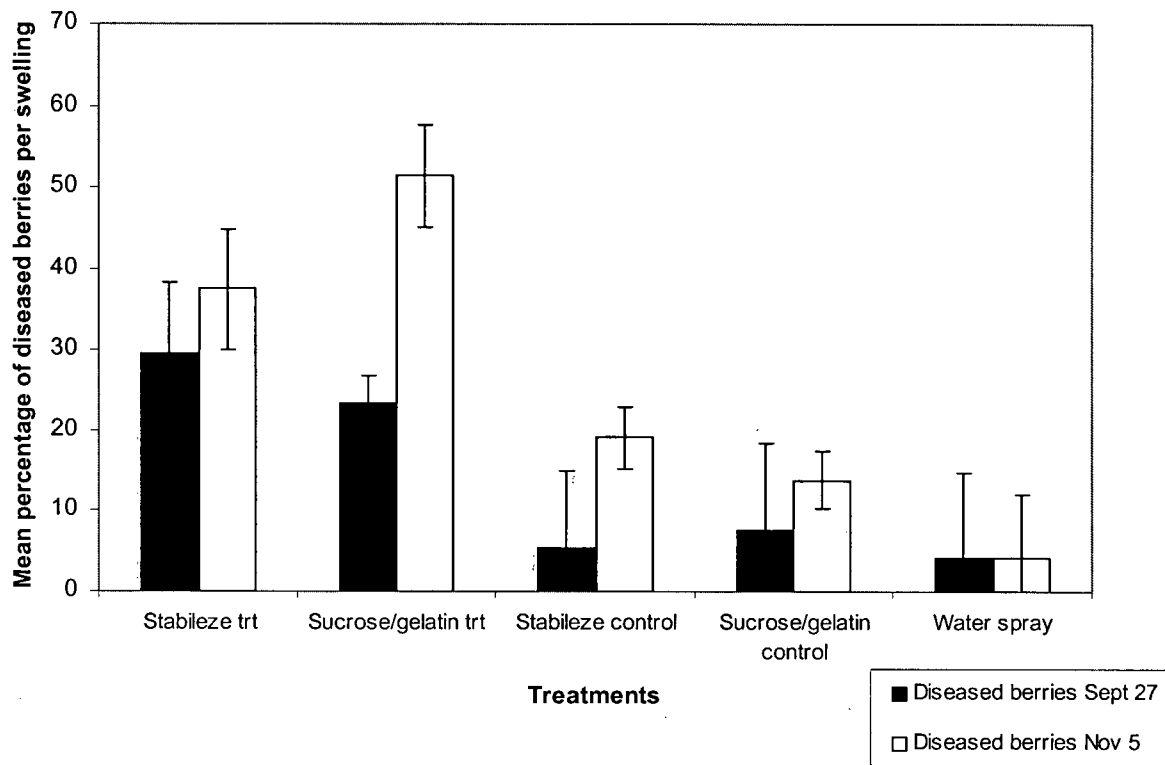
**Figure 3-8 Mean percentage diseased berries on September 27 and November 5, 2002 (day 102) as a percentage of total berries at the time of treatment. I-represents the standard error of the mean (S.E.)**

3.3.4 The assessment of *C. gloeosporioides* on shoot development

A repeated ANOVA test was applied to the shoot data for each month to compare differences in the number of diseased shoots for each treatment for each assessment. Table 3.6 outlines the number of healthy shoots counted on August 29, 2002 for each treatment. Table 3.7 and Figure 3.9 show the average of healthy shoots per swelling for each treatment after August 29, 2002. Analysis of the repeated ANOVA detected significant interaction between treatments and time (assessment date effect) and no significant differences between the treatments. A decline in shoots was observed for both treatments and controls over the 240 day period (Table 3.7 and Figure 3.9). The loss or

shedding of the dwarf mistletoe shoots for all treatments started after the assessment date September 12, 2002 or day 15.

A two-way repeated ANOVA detected no significant differences in the mean percentage of diseased shoots between the inoculum treatments and their respective control treatments for all the assessment dates. However, the assessment on September 27 (28 days after treatment), November (63 days after treatment) and December 18 (102 days after treatment) both inoculum treatments show a net loss in shoots when compared to their respective control and water control treatment. For the January (240 days after treatment) and February (172 days after treatment) there was a higher number of healthy dwarf mistletoe shoots for the 'Stabileze' treatment compared to its control. For the last assessment (240 days after treatment) there is net loss in shoots for all the inoculum treatment comparisons (net loss of 25.2% for 'Stabileze' and 9.5% for sucrose/gelatin).

True shoot analysis was difficult after 102 days due to the fact of high variation in number shoots within treatments for each assessment date and the reduction of healthy shoots for all the treatments over time.

Assessment of shoot losses before day 102, the 'Stabileze' treatment demonstrated the largest percent loss in dwarf mistletoe shoots at 28 days (26.4%) while the sucrose gelatin treatment demonstrate the largest losses in dwarf mistletoe shoots at 63 days (25.2%).

Table 3-6 Number of healthy shoots counted on August 29, 2002 (initial treatment date) for each treatment.

Treatments	Number of healthy shoots
Stabileze treatment	513
Sucrose/Gelatin treatment	556
Stabileze control	480
Sucrose/Gelatin control	499
Water spray	489

Table 3-7 Healthy dwarf mistletoe shoots as a percentage of the initial number of shoots from September 27, 2002 to May 15 2003 or 28 days to 240 days after treatment.

Assessment date	Days after treatment	Treatments-Percentage of healthy shoots				
		Stabileze	Stabileze control	Sucrose gelatin	Sucrose control	Water spray
Sept 15	0	100.0	100.0	86.0	98.0	86.0
Sept 27-	28	89.6	97.8	80.1	92.0	100.0
Nov 8	63	68.4	94.6	63.1	79.0	75.9
Dec 18	102	54.3	69.1	37.9	62.7	58.7
Jan 30	145	43.8	65.5	31.0	25.8	22.0
Feb 26	172	34.1	53.7	20.3	27.6	13.0
May 15	240	16.7	42.9	12.5	21.0	32.3
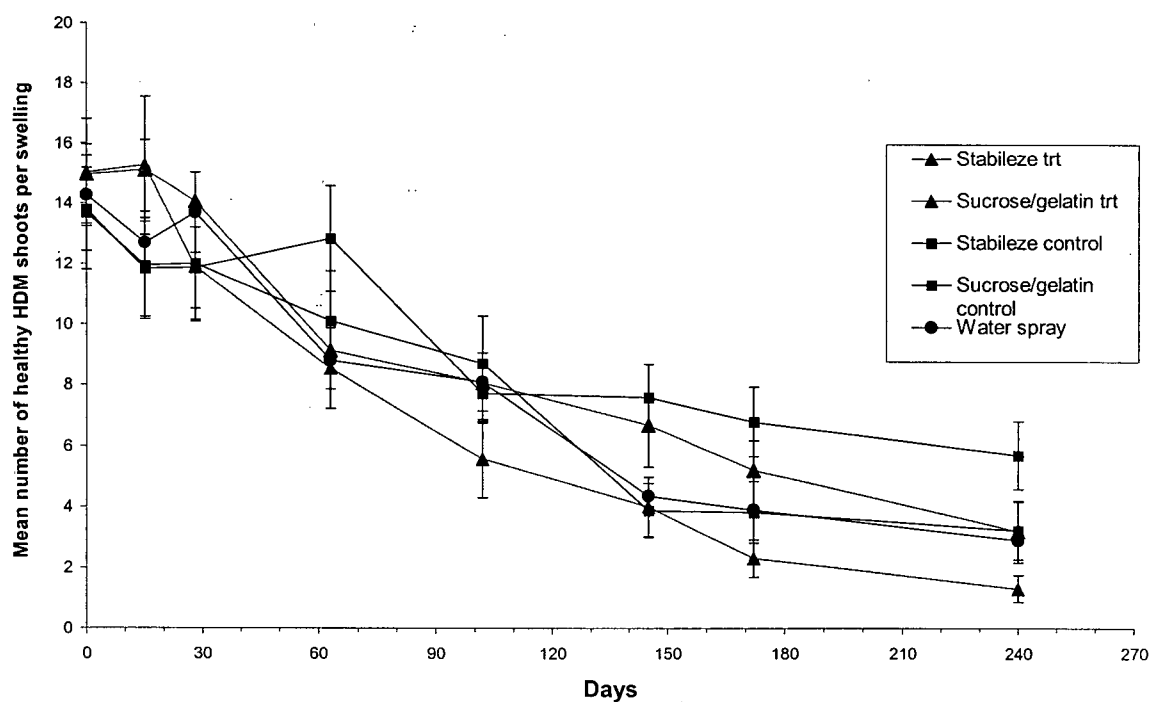
Figure 3-9 Average number of healthy shoots per swelling for each treatment after from August 29, 2002 (day 0) to May 12, 2003 (day 240). I-represents the standard error of the mean (S.E.)

3.3.5 Culturing from *A. tsugense* shoots

The results of isolation from asymptomatic and symptomatic are shown in Table 3.6. Dwarf mistletoe shoots were collected from intact treatments. Ten swellings were sampled from these treatments at the Spider Lake site. Three to 12 shoots per swelling were collected, labeled and categorized as symptomatic or non-symptomatic shoots for each treatment to determine the percentage of *C. gloeosporioides* that were re-isolated 10 months after the application of treatments (Table 3.6.). Symptomatic shoots from the control treatments were difficult to obtain.

Chi-square analysis detected significant differences in *C. gloeosporioides* reisolations between treatments and tissue grouping ($p < 0.01$). The symptomatic shoots of *A. tsugense* from the inoculum treatments showed higher recovery of *C. gloeosporioides* ($p < 0.01$) than non-symptomatic the shoots from the control treatments. Further analysis using the Fisher Exact test revealed that for the non-symptomatic shoot grouping for 'Stabileze' treatment and sucrose gelatin treatment demonstrated a significantly higher recovery of *C. gloeosporioides* reisolations compared to their respective controls ($p < 0.01$). A Fisher Exact test detected no significant differences for *C. gloeosporioides* recovery between the two inoculum treatments.

Table 3.7 outlines the percentage of unidentified fungi growing on PDA plates after 10 days aseptically culturing symptomatic and non-symptomatic shoots. This table demonstrates high percentage of fungi other than *C. gloeosporioides* re isolated from symptomatic and non-symptomatic shoots when cultured on the PDA plates.

Table 3-8 Percentage of reisolation attempts from symptomatic and non symptomatic shoots that yield *C. gloeosporioides* (May 21, 2003).

Treatment	Symptomatic shoots		Non-symptomatic shoots	
	Sample size	Percent of reisolation	Sample size	Percent of reisolation
Stabileze treatment	20.0	70	12	67
Sucrose/ Gelatin treatment	25.0	56	5	40
Stabileze control	3.0	0	37	3
Sucrose gelatin control	9.0	23	26	4

Table 3-9 Percentage of re-isolation from symptomatic and non-symptomatic shoots yielding one or more fungi other than *C. gloeosporioides* cultured on a PDA plate (May 21, 2003)

Treatment	Sample size-Symptomatic shoots	Percent of Symptomatic shoots	Sample size for non-symptomatic shoots	Percent of non-symptomatic shoots
Stabileze treatment	12	83.3%	20	95.0%
Sucrose/ Gelatin trt	5	100.0%	25	64.0%
Stabileze control	37	100.0%	3	100.0%
Sucrose gelatin control	26	100 %	9	100%

3.3.6 Destructive sampling of hemlock tissue infected with *A. tsugense*

Table 3.10 reveals the percent reisolations of *C. gloeosporioides* from various hemlock tissues infected with dwarf mistletoe. Chi squared test comparisons demonstrated that the inoculum treatments were significantly higher ($p< 0.01$) *C. gloeosporioides* recovery in the rhytidome compared to all the other infected hemlock tissues. 'Stabileze' cut shoot treatment was the only treatment in which *C. gloeosporioides* was recovered from the wood tissue (2 of 22 swellings).

Table 3-10 Percentage of reisolations of *C. gloeosporioides* from dead bark, living bark and dead wood within mistletoe swelling, 11 months (June 17, 2003) after the application of treatments.

Treatment	*Sample size	Percentage of re-isolation of *C. gloeosporioides*		
		rhytidome	Living bark	Wood
Stabileze treatment	9	45	0	0
Cut Stabileze treatment	22	50	32	14
Sucrose/ Gelatin treatment	7	72	15	0
Stabileze control	7	0	0	0
Cut Stabileze control	18	6	0	0
Sucrose/ Gelatin control	7	0	0	0
Water spray	13	8	0	0

- * The number of swellings examined for each treatment.

3.4 Discussion

Hemlock dwarf mistletoe spread is initiated by the forcible ejection of a viscous seed from dwarf mistletoe berry that can result in a dwarf mistletoe infection. After several years of incubation within the host, aerial shoots and flowers form resulting in the development of dwarf mistletoe berries (Hawksworth and Wiens 1996). Reducing the spread of dwarf mistletoe can be achieved by 1) reducing berries 2) reducing shoots and 3) killing the endophytic system of the dwarf mistletoe plant (Shamoun et al. 2003). In this field trial, the mean percentage of healthy berries was reduced by 16 % for the 'Stabileze' treatment and 36 % sucrose/gelatin treatment when compared with their respective control between 28 and 63 days after the application of treatments.

The primary and secondary cycles of infection by *C. gloeosporioides* that infects dwarf mistletoe is relatively unknown. In this study, acervuli were first observed in the field 4-8 weeks after the application of the inoculum treatments. A substantial increase in the number of diseased berries occurred between 28 and 63 days (Figure 3.1-3.5) while diseased shoots occurred between 15 and 28 days. The time required to form acervuli (1-2 months after application of treatments) runs parallel with the increase in number of diseased berries and reduction in the number of healthy shoots (Table 3.7 and Figure 3.9) which could indicate that secondary *C. gloeosporioides* infection can occur after 28 days. Appendix VII demonstrates that the relative humidity remained above 60% 28 days after treatments were applied. Studies on berry crops and fruit crops such as citrus fruit revealed that prolonged periods of wetness or following rainfall by rainfall promoted primary *C. gloeosporioides* infection (Avenlino et al. 2004). With blueberries and strawberries infected by *C. gloeosporioides* researchers have confirmed that secondary infection occurs after rainy, or misty or cool conditions (Freeman et al. 1998; Hildebrand et al. 2005). Makowsi and Mortensen (1998) have demonstrated with the biological control agent *C. gloeosporioides* that infects mallow, secondary infection (acervuli are capable of re forming) can occur after 5-7 days in favourable conditions. In the race of *C. gloeosporioides* that attacks blueberries and strawberries, spore dispersal peaked with the flowering and early fruit development (Wharton et al. 2004; Hildebrand et al. 2005). With *C. gloeosporioides* that attacks almonds, mummified fruit represents the main source of conidia for infection (Adskaveg et al. 1998; Wharton et al. 2004). For the *C.*

gloeosporioides that infects hemlock dwarf mistletoe, the primary and secondary infection, sources of inoculum, spread and persistence in the field has not been comprehensively investigated. Future research into the epidemiology of *C. gloeosporioides* will be important for the further development of this biological control.

The percentage and number of healthy dwarf mistletoe shoots decreased over the 240 day assessment period for both inoculum treatments and controls (Table 3.7 and Figure 3.9). The number of healthy shoots in the inoculum treatments was not statistically significantly different from their respective control treatments. Reductions in dwarf mistletoe shoots ranged from 24-26 % for both inoculum treatments, 28 to 102 days after the application of the treatments. A reduction in shoots was also noted during the last (after 240 days) assessment (8.5 to 26% percent loss in shoots for all treatments). The 'Stabileze' formulation inoculum treatment reduced the percentage of healthy mistletoe shoots more rapidly (63 days after treatments were applied) than the sucrose and gelatin inoculum treatment (102 days after treatments were applied).

The loss of shoots from the control treatments may have the result of *C. gloeosporioides* in the natural background (up to 23% C.g. isolation in the control treatments (Table 3.7)) and/or by secondary infections initiated by application of the inoculum treatments. In some cases, the control treatments were located adjacent to inoculum treatments increasing the possibility of secondary infection on the controls.

The reduction in mean percentage of healthy dwarf mistletoe shoots by the inoculum treatments may have been masked by decline in healthy shoots in all treatments after the November assessment date. Other possible explanation for low reductions in healthy dwarf mistletoe shoots includes 1) secondary *C. gloeosporioides* infection that may have spread to the control treatments, 2) a high variation in number of diseased shoots within and between treatments, 3) the number of diseased shoots that dropped off the swelling between assessments periods were not counted and 4) buds that developed into dwarf mistletoe shoots after August 29, 2002 assessment were not counted.

Probable causes contributing to dwarf mistletoe shoot loss during this assessment period include: 1) handling of experimental units at each assessment period and 2) environmental stress on the site that reduced the vigour of dwarf mistletoe plant resulting in shoot loss.

Overall reisolation success from *C. gloeosporioides* symptomatic shoots and berries in the field using the sterile technique was 83%. In some cases, no *C. gloeosporioides* was re isolated from diseased shoots. The reason for this could include (1) many different types of saprophytic fungi were reisolated from the diseased shoots and these fungi may have overgrown or out competed the *C. gloeosporioides* on the reisolation shoot plates, or (2) *C. gloeosporioides* no longer exists necrotic or dead symptomatic hemlock shoots.

Ten months after the treatments were applied to the swellings, symptomatic and non-symptomatic shoots were cultured from each treatment. Comparing all the treatments, symptomatic shoots showed a higher recovery of *C. gloeosporioides* and than the non-symptomatic shoots. Both symptomatic shoots and diseased berries may be sources of inoculum which may have spread to the control treatments. Studies with avocado, citrus, and mango show that infected leaves in the canopy were main sources of inoculum, with the conidia being rain-splash dispersed to unripe fruit (Wharton et al. 2004).

For the inoculum treatments, both the symptomatic shoots for the sucrose/gelatin and the 'Stabileze' treatments and the non-symptomatic shoots showed a higher recovery of *C. gloeosporioides* their control treatments.

Possible explanations for the high *C. gloeosporioides* recovery of non symptomatic shoots within the inoculum treatments is that this might be a result from secondary infection or that *C. gloeosporioides* occur as an endophyte on *A. tsugense C. gloeosporioides* commonly occurs as an endophytic fungus on several other plants (Bailey and Jeger 1992). For this study, it is highly unlikely that *C. gloeosporioides* remains an endophyte or latent in the non symptomatic shoots for 10 month period. The most likely scenario for the high recovery of *C. gloeosporioides* from the non symptomatic shoots was that secondary infections arising from the *C. gloeosporioides* treatments may have remained latent until environmental conditions were favourable for disease development. Fungal endophytes are fungi that live within the host plant either intracellularly by or intercellular resulting in non-symptomatic symptoms (Saikkonen et al. 1998). Pathogens and saprophytes can be fungal endophytes that have extended quiescent periods before disease or external signs of infection appear (Saikkonen et al. 1998; Redman et al. 2001). It has also been hypothesized that most endophytes are pathogenic fungi undergoing a latent phase of their lifecycle (Suske and Acker 1987;

Saikkonen et al. 1998). Further investigation into dwarf mistletoe *C. gloeosporioides* and its host relationship may determine the environmental and /or host physiological conditions that trigger the onset of the parasitic stage or prolonged saprophytic stage on dead tissue.

Latency can be defined as a quiescent or dormant relationship that after a definite period can beak down, causing disease (Makowski and Mortensen 1998). There is a possibility that this *C. gloeosporioides* may have latency characteristics. Latent infection of *C. gloeosporioides* has been reported on several plant species (Cerkauskas and Sinclar 1980; Hartman et al. 1986) and in green fruit (Dickman and Alvarez 1983; Prusky 1996; Bailey and Jeger 1992). Redman et al. (2001) suggests that pathogenic *Colletotrichum* spp. may have the ability to express different lifestyles based on the host genotype. Symbiotic lifestyles can be attributed to host fitness and a single *Colletotrichum* isolate may be pathogenic in one location and non-symptomatic in a different location (Redman et al. 2001).

Some researchers have suggested that *Colletotrichum* species can infect the endophytic systems of dwarf mistletoe. Parmeter et al. (1959) observed that *C. gloeosporioides* invaded the endophytic system of *A. campylopodum* f. *abietinum* on red fir. Wicker and Shaw (1968) observed *C. gloeosporioides* infection of the endophytic system of *A. americanum*, *A. campylopodum* f. *abietinum*, *A. larcis*, and *A. douglasii*. Ramsfield 2002; used histological techniques to concluded that there was no evidence that *C. gloeosporioides* invades the endophytic system of *A. americanum* in central British Columbia.

Destructive samples from dwarf mistletoe infected hemlocks from each treatment were used to determine if *C. gloeosporioides* invades the endophytic system. Precautions were taken to prevent the over-sterilization of the porous wood for the reisolation of *C. gloeosporioides*. The results of culturing the rhytidome, the living bark and woody tissue of western hemlock infected with *A. tsugense* from the inoculum treatments implied that the fungus rarely invades the endophytic system. Explanations for *C. gloeosporioides* for recovery in both living bark and wood for the 'Stabileze' cut shoots treatment were 1) biological control agent has easier access to wood through the basal cups from abscised

dwarf mistletoe shoot (Muir 1977) and 2) possibly a small piece of rhytidome remained attached to the living bark or wood samples.

Investigating the role of *C. gloeosporioides* on the infection process of *A. tsugense* may give an insight into the *C. gloeosporioides* biology and infection cycle. 'Stabileze' or sucrose and gelatin formulated with *C. gloeosporioides* do not appear to (enable the ability) of *C. gloeosporioides* to infect the endophytic system of dwarf mistletoe. The recovery of *C. gloeosporioides* from the destructive bark/wood tissue would imply that more than one application with these formulations *C. gloeosporioides* would be required to reduce dwarf mistletoe for an extended period of years using PFC 2415.

3.5   Future Work

3.5.1   Optimum conidia concentration of formulated *C. gloeosporioides*

The conidia concentration of the *C. gloeosporioides* used in this trial was 5.3×10^5 for the 'Stabileze", and 1.0×10^6 for sucrose gelatin mixture. Morin et al. (1996) found for *C. gloeosporioides* f. sp. *malvae* at a concentration of 2×10^7 conidia/mL was recommended to achieve adequate disease symptom to develop on the mallow leaves in the field. Other concentrations use for this biological control agent have been 5×10^5 conidia/mL for Johngrass, 8×10^7 conidia/mL for Sicklepod, 1×10^8 f conidia/mL or *Stylosathes guianensis* (Aubl.) Sw (Makowski and Mortensen 1998; Charudattan and Dinoor 2000; Charudattan 2001). The optimum *C. gloeosporioides* conidia concentration for infection appears to vary for different target plants. Future work may include determining an optimum concentration for *C. gloeosporioides* for an effective biological control agent.

3.5.2   Formulations for *C. gloeosporioides*

In this field trial a 'Stabileze' and a sucrose and gelatin formulation were use with PFC isolate 2415. Other formulations worth investigating for determining *C. gloeosporioides* efficacy is a soybean flour and sunflower oil mixture. This mixture increased the infectivity of *C. gloeosporioides* f. sp. *aeschynomene* on northern jointvetch (Sandrin et al. 2003). Formulations of spores in an inverted (water-in-oil) oil and water emulsions has been shown to reduce the need of long dew period of several similar biological control agents (Amsellem et al. 1990, 1991; Boyette et al. 1991, 1992; Ghorbani et al. 2000).

Sandrin et al. (2003) found that soybean and sunflower oil increased infectivity of *C. gloeosporioides* f. sp. *aeschynomene* to northern jointvetch by increasing spore germination, germ tube elongation and appressorium formation. Future work can include determining a formulation that will enhance efficacy in field situations.

3.5.3 Green fluorescent protein (GFP)

The green fluorescent protein (GFP) is widely used as a vital marker in many organisms, but is particularly useful in filamentous Ascomycetes. The GFP is a protein from the jellyfish *Aequorea victoria* that fluoresces green when exposed to blue light (Maor et al. 1998; Robinson and Sharon 1999). The jellyfish green fluorescent gene (gfp) is inserted into the *C. gloeosporioides* fungi resulting in the expression of the green fluorescence protein that can be visualized directly by a fluorescent microscope (Lee et al. 2002). The transformation of *C. gloeosporioides* can allow the researcher to identify *C. gloeosporioides* and determine the hemlock tissues it invades (Maor et al. 1998; Robinson and Sharon 1999). With careful sectioning of the infected swelling, fluorescent *C. gloeosporioides* infection can be tracked (personal comm. Dr S. F. Shamoun).

Due to the fact that transformed fungi produce copious amounts of spores, the application of such fungi to the dwarf mistletoe swelling must occur in a contained environment, such as in a lab, greenhouse or incubator, to prevent spread of this engineered gene to other wild type fungi (Loung et al. 2002).

The use of transformed fungus that produces GFP can be an useful tag for *in vivo* visualizing the infection process of biological control that does not require additional fixing of the host tissue (Maor et al. 1998; Robinson and Sharon 1999; Lorang et al. 2002). GFP *C. gloeosporioides* can also be used for other determinations such as the mode and speed of spread, evaluation and conditions for spread, further understanding the biology, fungal plant interactions, the efficacy and the fungal/endophytic fungal reactions of *C. gloeosporioides* that attacks *A. tsugense* (Loran et al. 2002).

The green fluorescent protein (GFP) is widely used as a vital marker in many organisms, but is particularly useful in filamentous Ascomycetes. DNA from the GFP is inserted into the biological control of interest, in this case *C. gloeosporioides* that infects *A. tsugense,* allowing the researcher to identify *C. gloeosporioides* and determine the

hemlock tissues it invades (Maor et al. 1998; Robinson and Sharon 1999). The transformed fungi can be identified using a fluorescent microscope (personal comm. Dr S. F. Shamoun). The fluorescent *C. gloeosporioides* is usually applied under controlled conditions (such as in a greenhouse or incubator) to an *A. tsugense* swelling. With careful sectioning of the infected swelling, fluorescent *C. gloeosporioides* infection can be followed. The use of fungus DNA with GFP can determine the mode of action of this biological control (Maor et al. 1998; Robinson and Sharon 1999). GFP *C. gloeosporioides* can also be used for other determinations such as the mode and speed of spread and conditions for spread further understanding the biology, fungal plant interactions and the fungal/endophytic fungal reactions of *C. gloeosporioides* that attacks *A. tsugense*.

3.5.4 Modeling

The Forest Vegetation Simulator (FVS) model can assist in determining the impact of dwarf mistletoe (*A. tsugense*) on a stand. This model can simulate several types of different harvest regimes that will indicate significant future forest harvests (Robinson et al. 2000, 2003; Muir 2003) and assist foresters and others to determine acceptable levels of dwarf mistletoe damage under different environmental conditions and forest practices (Robinson et al 2002; Muir et al. 2004).

Biological control effects of *C. gloeosporioides* on dwarf mistletoe can be inputted into the FVS model allowing for projected scenarios of the spread and intensification of the treated dwarf mistletoe stand. If the effects on reduction of shoots and berries by the biological control agent can reduce the dwarf mistletoe to an acceptable level (e.g., significantly increase the hemlock growth and lower the dwarf mistletoe rating), it may be beneficial and cost effective to apply the biological control agents. As noted in Chapter 1 work on this model is temporally impeded due to lack of funding.

3.5.5 Combination of *C. gloeosporioides* and *Neonectria neomacrospora*

Perhaps the most promising biological control for *A. tsugense* would consist of combining *C. gloeosporioides* and *Neonectria neomacrospora*. The faster-acting *C. gloeosporioides* fungus has the ability to reduce the shoots and berries within 2 to 3

months after application while the slower-developing *N. neomacrospora* fungus may girdle the host after a year with wounded or longer in unwounded infected hemlock branches (Rietman 2004). Harvey et al. 1996 used a combination of *C. gloeosporioides* and *Pleiochaeta setosa* (Kirchner) Hughes 3×10^5 and 2×10^4 conidia/ml respectively to control Russell lupine. The addition of *P. setosa* conidia to the inoculum increased the level anthracnose with Russell lupine (Harvey et al. 1996). The combination of *C. gloeosporioides* and *N. neomacrospora* may also increase the level of control for dwarf mistletoe.

Both biological control agents are easy to culture, virulent to shoots and berries or capable of girdling the infected branch of *A. tsugense*. A combination of both the biological control agents may be effective in reducing dwarf mistletoe for a year or longer depending on the efficacy of the *N. neomacrospora* (Rietman 2004; Shamoun et al. 2003). If both biological control agents for dwarf mistletoe are compatible including increasing both their efficacy potentials, it could be possible that one time spray application could reduce dwarf mistletoe.

3.5.6 Need for biological controls

Large-scale clear cutting and removal of dwarf mistletoe residuals has been standard practices for timber harvest in B.C. Recently the B.C forest service have reduced clear-cut harvesting and switched to variable retention. Variable retention harvest systems results in maintaining as many mature trees as possible to maintain structural elements (personal comm. B. van der Kamp 2006) over the area of the block for at least one rotation (Mitchell and Beese 2002). The combination of opening up the stand and maintaining dwarf mistletoe infected mature trees throughout the harvest block increases the probability of infecting the majority of the newly regenerating stand with dwarf mistletoe (Edwards 2002; personal comm. B. van der Kamp 2006). Variable retention harvest systems can limit the methods for traditional silviculture control (Shamoun et al. 2003). Chemicals that control dwarf mistletoe are not cost effective and the use of genetically resistant hemlock trees has not yet been developed for the control of dwarf mistletoe (Shamoun et al 2003; Cartwright et al. 2004; personal comm. B. van der Kamp 2006).

The dwarf mistletoe simulator can be amended to simulate the effects of *C. gloeosporioides* and *N. macrospora,* applied to a dwarf mistletoe infected stand, by calculating the reduction in dwarf mistletoe seed production (Robinson et al. 2002; Muir 2003). These amendments to this model will allow stakeholders to determine the long term effects of biological control interventions. Important parameters used to predicting the degree of dwarf mistletoe control include the efficacy of the agent, the intensity of dwarf mistletoe infection in the stand and variety of stand conditions (such as spatial arrangement of trees, site location, tree species, light environment and tree canopy) (Robinson et al. 2002). This model may be used as a technique to predict the most cost effective method of controlling dwarf mistletoe for a particular stand. In stands where silvicultural practices are restricted, biological control agents may be the only viable method of reducing the impact of dwarf mistletoe.

Most of the proposed research on *C. gloeosporioides* is ongoing in the laboratory of Dr. S.F. Shamoun at the Canadian Forest Service, Pacific Forestry Centre, Victoria along with the collaborative work with the British Columbia Forest Service, Wood Science Department at University of British Columbia, Dr. John Muir, Head Forest Pathologist at BC of Forests (retired) Ministry of Forest and Range, Research Branch, Coastal Tree Breeding Program and Don Robinson at ESSA Technologies Ltd.

3.6 Conclusion

From this field study, *C. gloeosporioides* inoculated on *A. tsugense* swellings initiated disease within the first 3–4 months after inoculation for both the berries and shoots. *C. gloeosporioides* was effective in reducing the number of healthy berries. While the results for shoots reduction appeared promising, further proof is required to establish *C. gloeosporioides* can significantly reduce dwarf mistletoe shoots. The effects of *C. gloeosporioides* on dwarf mistletoe shoots in this study may have been masked by the heavy background infection and/or secondary infection.

Two months and ten months after treatments were applied the recovery of *C. gloeosporioides* from the dwarf mistletoe shoots were higher on the inoculum treatments (including the non-symptomatic shoots) than the control treatments. The latency period of *C. gloeosporioides* that attacks hemlock dwarf mistletoe has not been determined for this

biological agent. Determining triggers that may activate the necrotrophic phase may be a key to enhancing the efficacy of *C. gloeosporioides* present in dwarf mistletoe shoots.

Destructive sampling revealed that *C. gloeosporioides* does not infect the living bark and secondary wood of *A. tsugense* unless shoots are abscised or cut near the base of the basal cup. The use of GFP may determine the mode of infection for this fungus.

To increase the reduction of dwarf mistletoe aerial shoots and berries by *C. gloeosporioides* future research should include: screening large number of hemlock dwarf mistletoe *C. gloeosporioides* isolates from different geographical location to select more virulent isolates; establishing the optimum concentration of the conidia for effective dwarf mistletoe infection, selecting the most compatible formulation and to determine combinations of other biological control agents to increase the efficacy of *C. gloeosporioides*.

Determining the *C. gloeosporioides* lifecycle on dwarf mistletoe such as methods of conidia spread, secondary infection and latency tendencies (if they exists) and the location of the overwintering stage of the conidia (upper or lower canopy or shoot litter found on the ground) also needs further investigation. Insights for optimum timing of spraying and predicting optimum environmental conditions for disease outbreaks of *C. gloeosporioides* can further increase the efficacy of this biological control agent.

The most important objective of this study was to evaluate *C. gloeosporioides* as a biological control for hemlock dwarf mistletoe. Data collected from this study determined that an application of this biological control agent decreased the number of dwarf mistletoe berries and may have some influence in the reduction of the number of healthy dwarf mistletoe shoots. Further studies are required to determine the effect of secondary infection and persistence of *C. gloeosporioides* in field conditions. To develop *C. gloeosporioides* into effective and possibly a commercially available biological control product for dwarf mistletoe control there are number of challenges that need to be addressed.

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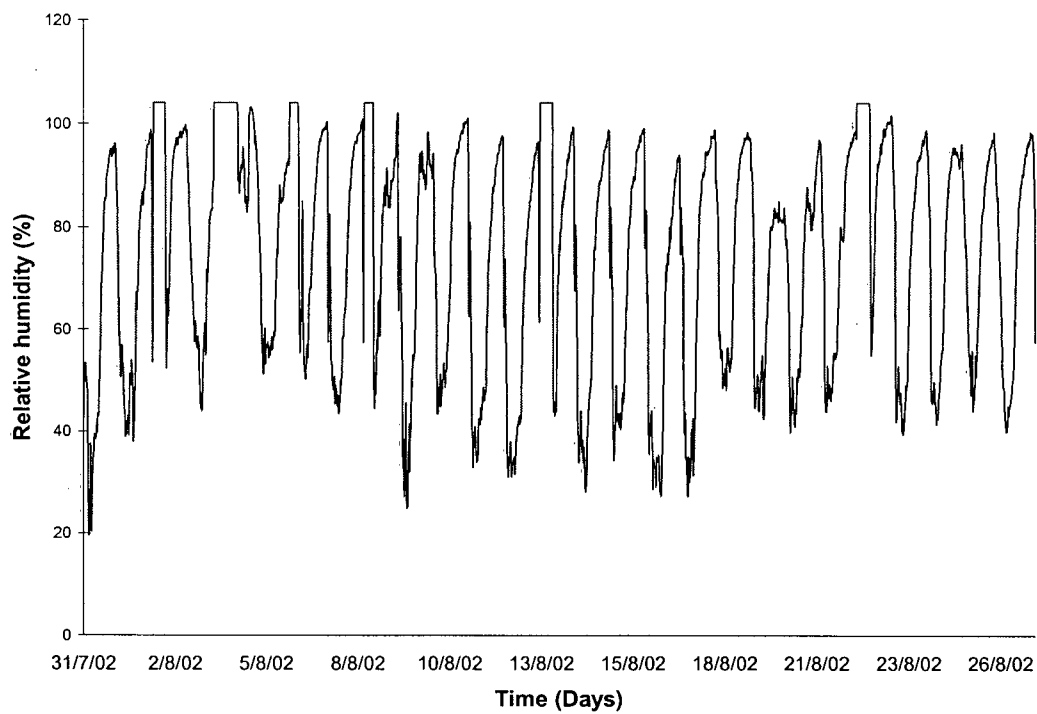
APPENDICES

PFC site
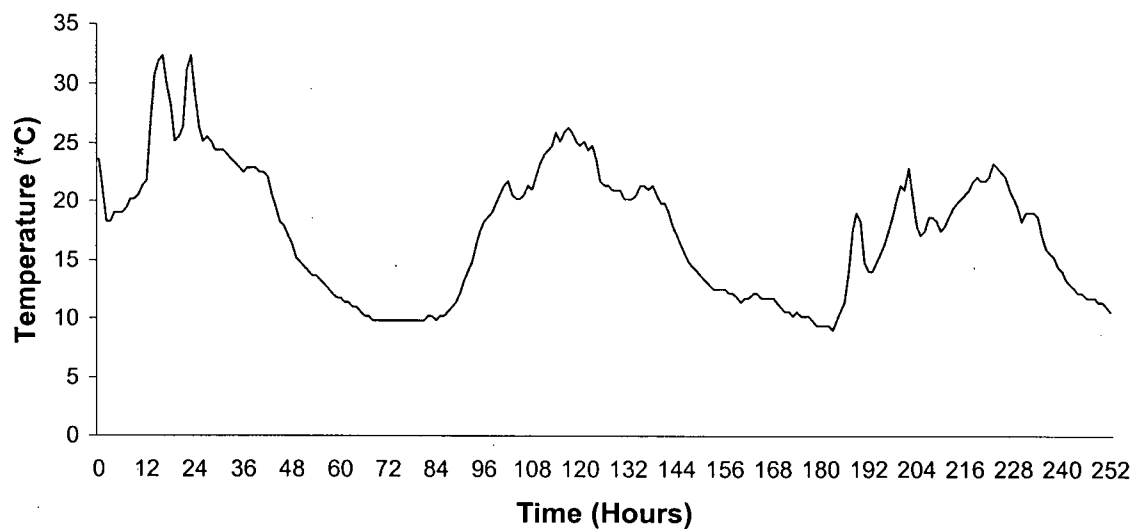
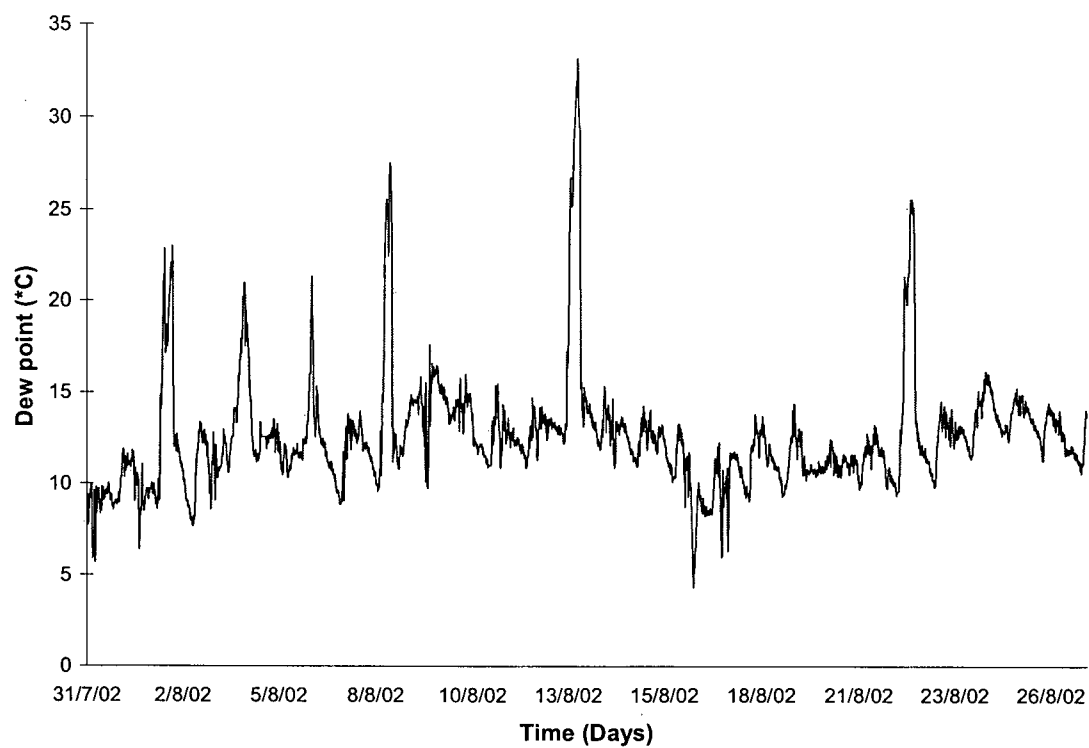**Appendix I Temperatures (°C) at the PFC site after the application of the treatments (in hours)**
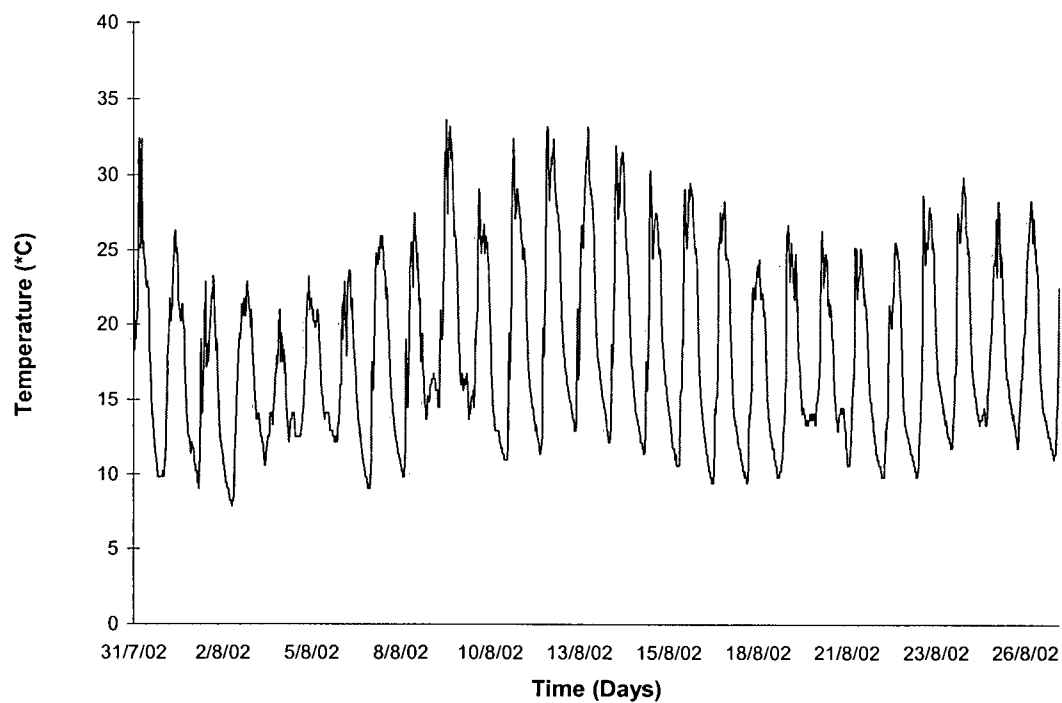
Appendix II Temperatures (°C) at the PFC site after (in days) the application of the treatments
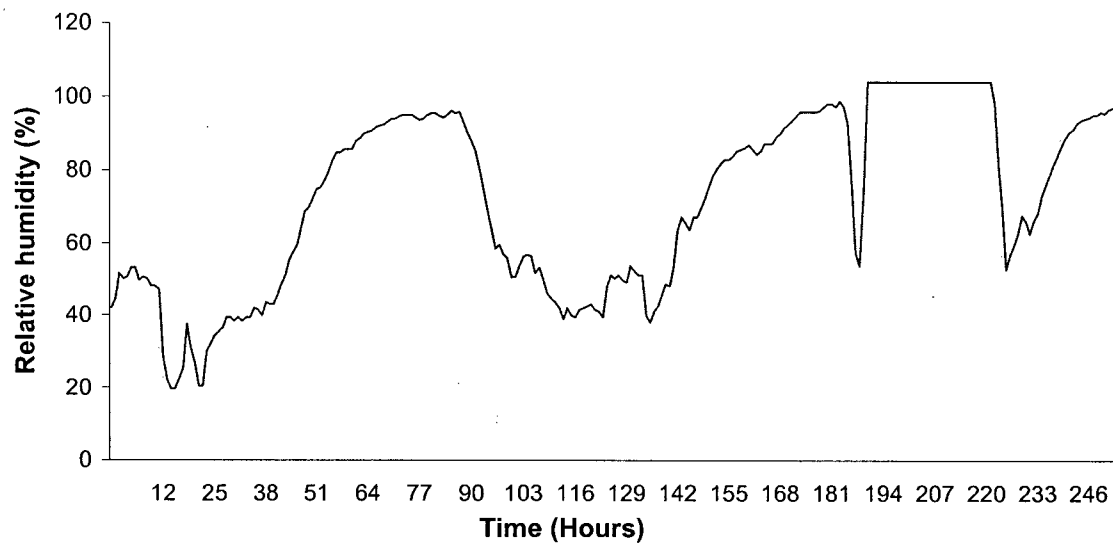
Appendix III Relative humidity (%) at the PFC site after (in hours) treatments were applied.
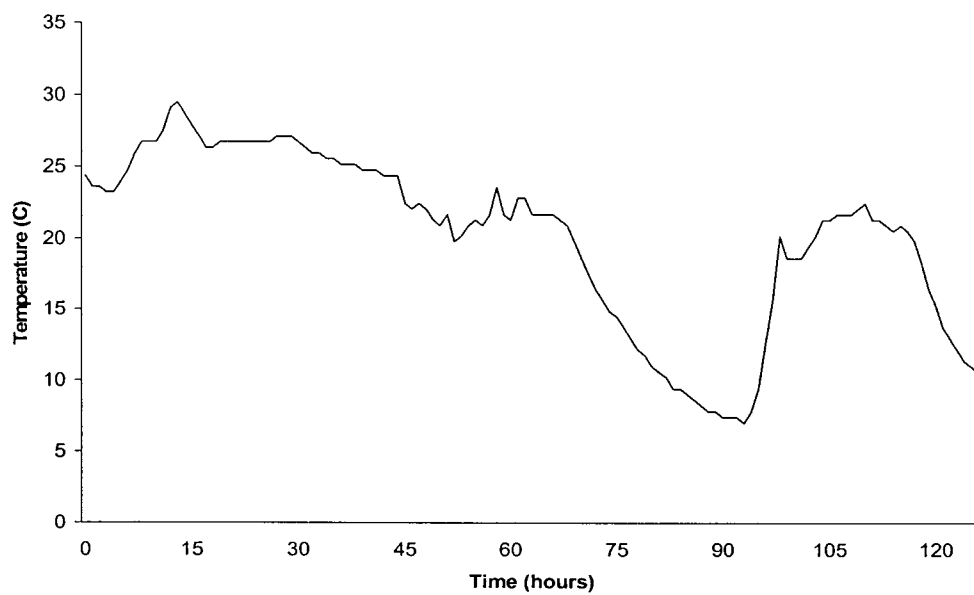
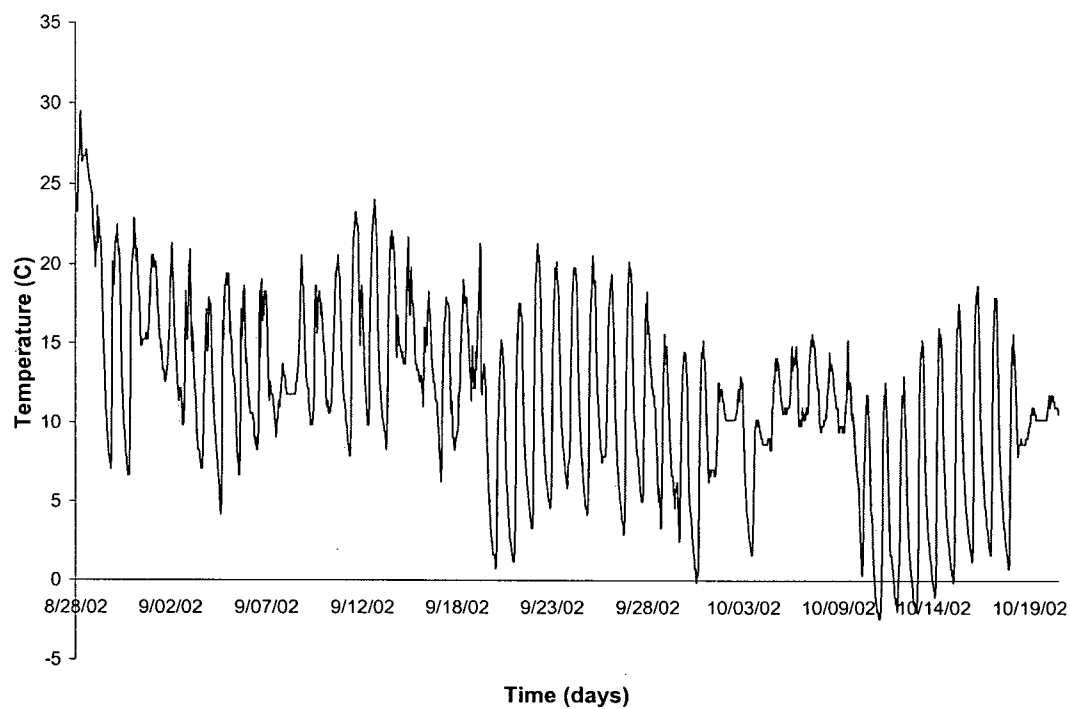**Appendix IV Relative humidity (%) at the PFC site after (in hours) treatments were applied.**

Appendix V Dew Point at the PFC site after (in days) the treatments were applied.
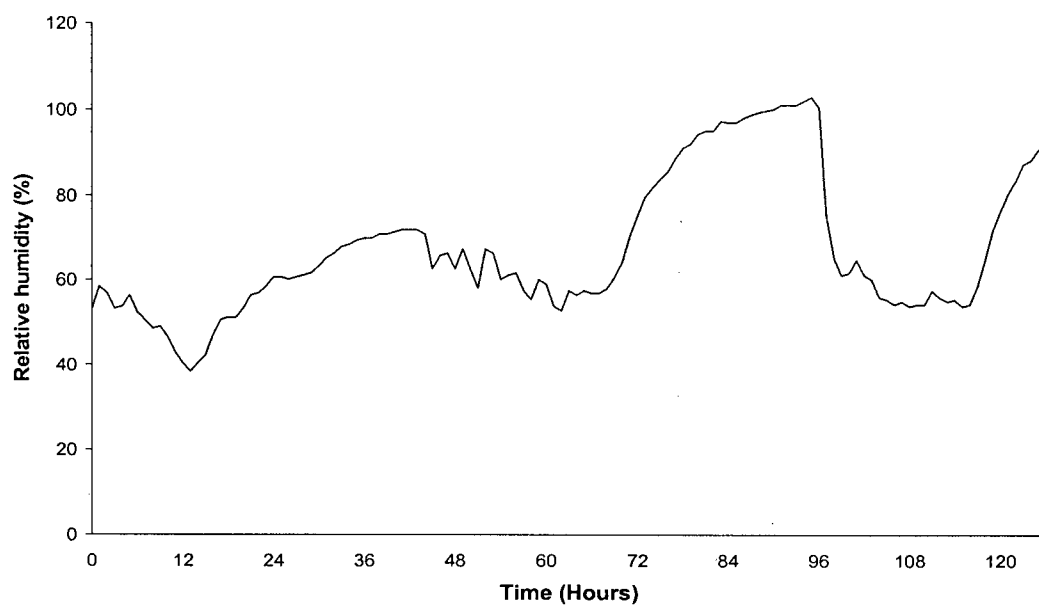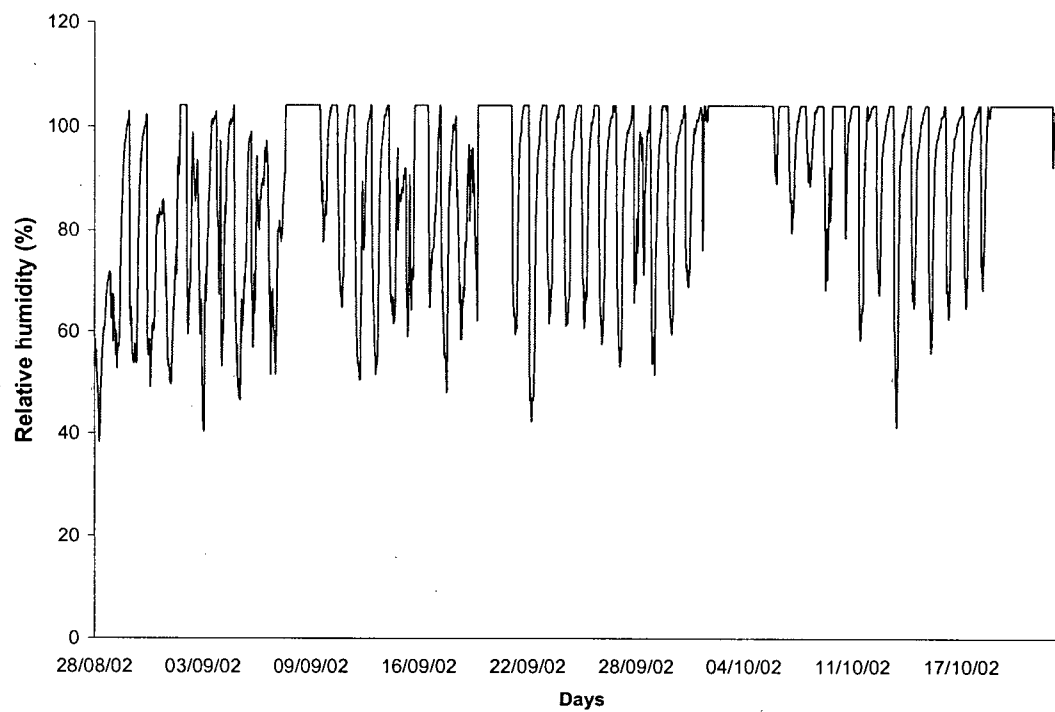
Spider Lake site

Appendix VI Temperatures (^{o}C) at the Spider Lake site after (in hours) the application of treatments
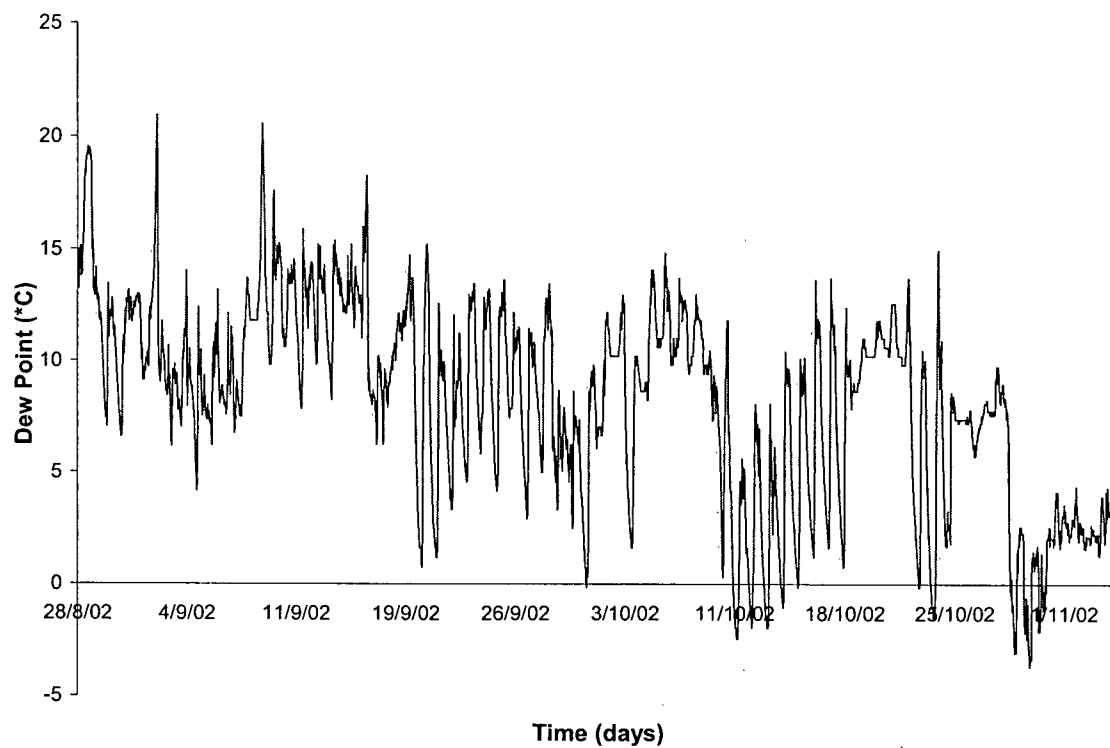
Appendix VII Temperatures (^{o}C) at the Spider Lake site after (in days) the application of treatments

Appendix VIII Relative humidity (%) at the Spider Lake site after (in hours) the application of treatments.

Appendix IX Relative humidity (%) at the Spider Lake site after (in days) the application of treatments

Appendix X Dew points at the Spider Lake site after (in days) the application of treatments.