# GENETIC DIVERSITY AND POTENTIAL BIOLOGICAL CONTROL OF *RUBUS SPECTABILIS*

by

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**ABSTRACT**

A mycobiota survey was conducted on Vancouver Island and mainland BC to identify potential biocontrol agents of salmonberry. The species, which included 17 isolates of *Botrytis cinerea*, 3 *Phoma argillacea* and 1 *Septoria rubi*, were pathogenic on detached leaves. Greenhouse trials showed *P. argillacea* induced foliar and stem necrosis on intact plants with an 18-hr leaf wetness period and mycelial inoculum of $2.6x10^4$ CFU/mL. In a host range test, three conifer species showed seedling tolerance and red raspberry showed initial damage but no cane blight when inoculated with *P. argillacea*. Salmonberry DNA was isolated from leaves collected from five widespread populations. RAPD-PCR was used to fingerprint individuals, generating 35 loci with a 75.5% average variability. Diversity within populations was high, with an average heterozygosity of 0.38. Among population differentiation was poor, with an $F_{ST}$ of 0.0454; therefore, an average 5.3 migrants per generation is required to maintain this variation.

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## 1.1. *Rubus spectabilis*

### 1.1.1. Introduction

*Rubus spectabilis* Pursh. (Rosaceae), commonly known as salmonberry, is a native deciduous perennial shrub of western North America. It is commonly a dominant understory species in natural forests, as well as a pioneer species that rapidly invades disturbed forests and roadside areas where the forest canopy has been opened through logging. *Rubus spectabilis* forms dense, multilayered and monospecific stands of extensive clonal colonies. *Rubus spectabilis* provides food and habitat for several forest fauna and plays a role in nutrient cycling and reduction of soil erosion. However, *R. spectabilis* is also considered a forest weed in conifer regeneration sites and can negatively impact young conifer seedling growth through competition for nutrients, light and water. Its origin and distribution, life history and biology, taxonomy and phylogeny and diversity, ecological role and uses, management and control, and natural diseases are reviewed in this section.

### 1.1.2. Origin and distribution

Southwestern China may be the center of origin for the genus *Rubus* as it is geologically archaic and was not covered by glaciers during the Quaternary (Gu et al. 1993). This region contains a high diversity of *Rubus* species, having at least 299 botanical taxa distributed in 27 provinces (Gu et al. 1993), mostly belonging to the subgenera *Idaeobatus* and *Malachobatus* (Lu 1983), as well as a high number of subgenera and morphological variation (Lu 1983). The two largest and most important subgenera in North America are *Eubatus* (blackberries) and *Idaeobatus* (raspberries)

(Alice and Campbell 1999). Fossil records suggest that the subgenus *Idaeobatus* has its center of origin in eastern Asia (Barber 1976). However, a phylogenetic study of the genus *Rubus* based on ITS ribosomal DNA data suggest a western North American or far eastern Asian origin (e.g., Japan or eastern Russia) (Alice and Campbell 1999).

Fossil records from the Tomales Bay region in northern California indicate that salmonberry was present there during the Pleistocene period (Barber 1976). The first botanical record of *R. spectabilis* in North America was in *Flora Americae Septentrionalis* in 1814 (Hitchcock et al. 1961) when it was discovered and named by Pursh. Long before this, the Native Peoples used *R. spectabilis* and other *Rubus* species primarily as food plants as fruits and shoots were gathered in abundance (Pojar and McKinnon 1994). *Rubus spectabilis* is native to North America and its natural range extends west of the Coast Mountains, British Columbia from low subarctic/high temperate regions in the Aleutian Islands and southern Alaska, south to northwestern California (Oleskevich 1996). It has also been reported to reach as far as Yezo (Hokkaido) and Honshu Islands in Japan (Barber 1976). *Rubus spectabilis* is commonly found along the west coast of British Columbia and inland along the Skeena and Fraser River drainages (Haeussler et al. 1990). Its elevation ranges from sea level to lower alpine elevations (Hulten 1974), but it is most abundant below 800 m (Barber 1976).

In British Columbia, *R. spectabilis* grows well in both old growth and forest regeneration sites. The spread of *R. spectabilis* is limited by light, moisture, intolerance to cold temperature and short growing season (Klinka et al. 1989). However, it has a high shade tolerance and can achieve maximum photosynthesis at lower light radiation levels (150 $\mu E\ m^{-2}s^{-1}$) (Barber 1976). *Rubus spectabilis* also grows on a wide range of

soil types, with optimum growth occurring on floodplains with well-aerated soils near field capacity (Haeussler et al. 1990) and is an indicator plant species of nitrogen-rich forest soils (Klinka et al. 1989). *Rubus spectabilis* is a pioneer species found in open forest sites disturbed by logging, fire or silvicultural activities along the coast of BC under old- and second-growth forests (Klinka et al. 1989). The current increase in spread and distribution of *R. spectabilis* is primarily attributed to logging and land clearing practices implemented in the last century (Oleskevich 1996), coupled with its prolific formation of extensive clonal colonies. *Rubus spectabilis* is considered a weedy shrub in forest regeneration sites in western North America requiring intensive vegetation management using both conventional (manual brushing and chemical) and alternative (grazing and biological control) methods of control.

Outside of its natural distribution range, *R. spectabilis* was brought to the British Isles in 1827 and introduced as pheasant food in Scotland. It was originally introduced as an ornamental or hedge plant but has become widespread and weedy in the Orkneys and recent investigations have suggested that *R. spectabilis* should be considered a potential pest in forestry plantations in Northern Ireland (Paterson and Binggeli 1995).

### 1.1.3. Life history and biology

*Rubus spectabilis* is a perennial deciduous shrub, which contains remarkable morphological variation. It has erect or curved stems arising from extensive branching rhizomes, compound mostly trifoliate leaves, solitary flowers that are pink to reddish-purple in color, and mature yellow to glossy red fruits formed as an aggregate of small drupelets (Hitchcock et al. 1961; Taylor 1973). Shrub height has been observed to reach as high as 2 m in reforested areas in British Columbia.

Flowering occurs early in spring with initial bud burst and leaf flush occurring in March-April and April-May, respectively. Leaf expansion is often complete by May with senescence and leaf drop occurring in late October (Maxwell et al. 1993). Seeds germinate by early April with new seedlings appearing in early June (Ruth 1970).

Flowers are self-infertile (Keep 1968) and seeds are produced through cross-pollination followed by fertilization with normal embryo development (Virdi and Eaton 1969a). In general, species in the subgenus *Idaeobatus* are diploid; the chromosome number of *R. spectabilis* is 2n = 14 (Thompson 1995), also diploid. Apomixis, the production of seeds without meiosis or fertilization, is common in *Rubus* subgenus *Rubus* (blackberries) (Nybom 1988) but rarely occurs in *Rubus* subgenus *Idaeobatus*, only occurring in a few triploid specimens (Jensen and Hall 1979). *Rubus spectabilis* flowers are pollinated by unspecialized insects such as beetles (Barber 1976). Salmonberry fruits are dichromatic, producing orange (formerly called 'gold') in addition to red fruits (Traveset and Willson 1998; Willson 1994; Burns and Dalen 2002). The orange colouration of fruit produced by *R. spectabilis* may be due to the predominance of pelargonin glycosides (Oleskevich 1996) and the red colouration is due to an abundance of the anthocyanin pigments and the darkness of the red is somewhat variable depending on the amount of pigment (Griffiths and Ganders 1983).

Seed production in *R. spectabilis* is through sexual reproduction; the species produces over 300,000 seeds per kg (Tappeiner and Zasada 1993). Dispersal is either directly below the parent plant (as ripe fruit falls), throughout the soil by burrowing animals, or by fruit consumption and dispersion by birds and mammals (Oleskevich 1996). Seed dispersal is the primary means by which *R. spectabilis* colonizes new sites,

with seed viability remaining for at least 100 yr (Oleskevich 1996). Seeds germinate following scarification in sulfuric acid, cold stratification and alternating temperatures at 2-3°C for 5 months (Barber 1976; Tappeiner and Zasada 1993).

*Rubus spectabilis* also spreads through vegetative reproduction through extensive clonal colonies thought to be responsible for the establishment of *R. spectabilis* in new environments. Vegetative growth creates lineages of genetically identical offspring. Since *R. spectabilis* exists at wide elevation ranges and ecosystems, clonal growth is particularly advantageous in preserving genotypically identical lineages of *R. spectabilis* that are well-adapted to the immediate environment. *Rubus spectabilis* shrubs have an extensive rhizome system with annual ramets arising from a large rhizomal bud bank (Zasada et al. 1992; 1994) and produce about 1-2 new rhizomes/yr extending 0.1-0.8 m/yr (Tappeiner et al. 1991).

### 1.1.4. Taxonomy, phylogeny and diversity

The genus *Rubus* (Rosaceae) is composed of both herbaceous and woody species (Barber 1976). It is a large genus consisting of several hundred sexual species to thousands of apomictic species (Morden et al. 2003) with approximately 740 species described throughout the world (Gu et al. 1993). However, the exact number of species is unknown because a worldwide taxonomic treatment of the genus has not appeared since Focke (1910, 1911, 1914) and does not include more recently described species (Thompson 1995). *Rubus* species are found on all arable continents from the lowland tropics to subarctic regions (Thompson 1995) at elevations from sea level to 4500 m (Hummer 1996), with the exception of Antarctica (Alice and Campbell 1999). *Rubus* is divided into12 subgenera by Focke (1910, 1911, 1914) with numerous sections and series

(Alice and Campbell 1999). The two largest and most important subgenera in North America are the raspberries (subgenus *Idaeobatus*) and the blackberries (subgenus *Rubus* = *Eubatus* Focke) (Alice and Campbell 1999). Originally, 117 species were placed in the subgenus *Idaeobatus* by Focke, but this has since risen to 135 species (Thompson 1997). The greatest expansion in species number is found in the subgenus *Rubus* increasing from 132 to 400 sexual species and thousands of apomictic species (Jennings 1988; Morden et al. 2003). Phylogenetic treatment of this genus has primarily been based on morphology and some cytogenetic study to determine ploidy level. However, there are levels of homoplasy and plasticity in key morphological characters such as leaf type and stem armature, encouraging the use of molecular data. More recent phylogenetic studies based on molecular markers such as ribosomal DNA, chloroplast DNA and ITS show that *Rubus* subgenus *Idaeobatus* is polyphyletic providing phylogenetic relationships among the different *Rubus* subgenera as well as information on divergence (Howarth et al. 1997; Morden et al. 2003; Alice and Campbell 1999). The length and GC content of ITS 1 and ITS 2 of the 56 *Rubus* species tested by Alice and Campbell (1999) are similar to other reported angiosperm sequences and therefore they concluded that nucleotide substitution appears to be the main source of variability.

The common name of *R. spectabilis* is salmonberry (Taylor and McBryde 1977). It is believed that the common name is derived from the use by the Chinook Indian tribes of the Pacific Northwest of the salmonberry bark as a remedy for indigestion caused by eating too much salmon (Barber 1976). There are no reported varieties of *R. spectabilis* subspecies in Canada although var. *franciscanus* (Rydb.) (syn. var. *menziesii* (Hook.) S. Watts (Kartesz 1994) has been reported in California and ssp. *vernus* Focke is reported in

Japan (Barber 1976). *Rubus spectabilis* bears a close morphological resemblance to *R. hawaiiensis* (Gardner et al. 1997) and is thought to share a common ancestor (Gardner et al. 1997). Recent molecular studies using the choloroplast gene *ndhF* (NADH dehydrogenase) (Howarth et al. 1997; Morden et al. 2003) also strongly support the close relationship between *R. hawaiiensis* and *R. spectabilis*.

There are no studies on clonal genetic diversity of *R. spectabilis* in its natural range. Therefore, the present study will provide relevant information on *R. spectabilis* genetic diversity in British Columbia. Phenotypic expression of *R. spectabilis* genetic diversity has been observed through fruit color and flower variations. As previously mentioned, *R. spectabilis* fruits are either red or orange. Sometimes, *R. spectabilis* fruits have so much anthocyanin pigments that they appear purplish in colour. Color differences can arise from relatively simple mechanisms, such as the attachment of a particular molecule to a pigment precursor (Traveset and Willson 1998). Fruit color is probably controlled by a single gene with the allele for red fruits dominant and the allele for orange fruits recessive (Griffiths and Ganders 1983). This pattern is also observed in the close relative of *R. spectabilis*, the cultivated *R. idaeus* (red raspberry), but the orange fruit allele is not nearly as common in the raspberry as in *R. spectabilis* (Griffiths and Ganders 1983). This polymorphism is found throughout the geographical distribution of *R. spectabilis* (Gervais et al. 1999) and in British Columbia, *R. spectabilis* populations are polymorphic. A study by Gervais et al. (1999) found that the orange fruit morph of *R. spectabilis* occurred more frequently in Southeast Alaska than the red fruit morph. However, the function of fruit polymorphism is not well-studied. One of the most plausible hypotheses explaining the evolution of color in fleshy fruits is that this trait

responds to selective pressures exerted by fruit eating animals (frugivores). In *R. spectabilis*, it has been suggested that avian foraging behavior may help maintain the color polymorphism (Traveset and Willson 1998; Gervais et al. 1999).

In addition to fruit color variation, *R. spectabilis* also exhibits flower shape diversity. *Rubus spectabilis* flowers typically have five petals and numerous stamens but the double-flowered mutants have numerous petals so that the stamens and pistils at the center of the flower are barely visible (Figure 1.3) (Griffiths and Ganders 1983). These extra petals are essentially modified stamens and are a common mechanism by which double flowers are formed (Griffiths and Ganders 1983). Double-flowered *R. spectabilis* is cultivated and utilized as ornamentals at various locations such as Heritage Court near the Provincial Museum in Victoria, University of British Columbia Botanical Garden, and University of Washington Arboretum (Griffiths and Ganders 1983). White-flowered *R. spectabilis* has also been known to occur in nature. The only white-flowered *R. spectabilis* known is a single plant that occurs in Cathedral Grove, MacMillan Park, near Port Alberni, on Vancouver Island (Griffiths and Gander 1983), the genetics of which has not yet been studied, but is likely the result of a single recessive gene.

### 1.1.5. Ecological and other uses

Although regarded mainly as a weedy species, *R. spectabilis* is an important plant in nutrient cycling and conservation (Oleskevich 1996). It serves as forage and cover for wildlife such as elk, deer, moose and mountain goats (Barber 1976). Coyotes, bears and birds eat the fruits, while flowers supply nectar to hummingbirds, bees and other insects (Barber 1976). *Rubus spectabilis* is an important part of the spring and summer diet of coastal black and grizzly bears (Lloyd 1979) and a portion of the Roosevelt elk's summer

diet (Jenkins and Starkey 1991). Young sprouts of *R. spectabilis* have been utilized as food by Coastal Native Peoples including the Nuu-chah-nulth, the Kwakwaka'wakw and Nuxalk (Turner 1975). Berries of *R. spectabilis* were often eaten fresh, sometimes with salmon (Oleskevich et al. 1996). Although not as tasty as *R. strigosus* (wild red raspberry), *R. spectabilis* fruits are valued by berry-pickers.

In addition to its role as a food source for humans and wildlife, *R. spectabilis* retards soil erosion because of its extensive system of roots and rhizomes (Barber 1976) and is often planted in reclamation sites in avalanche areas, bank stabilization along steep road cuts and streams, and for dune stabilization (Hungerford 1984; Marchant and Sherlock 1984; Minore and Weatherly 1994). Economically, flowers and foliage of *R. spectabilis* are considered somewhat valuable (Taylor and McBryde 1977). Because *R. spectabilis* easily hybridizes with *R. idaeus*, it is valued for crossing and cultivation of red and black raspberries plants (Virdi and Eaton 1969b; Jennings and Ingram 1983; Daubeny and Anderson 1993). It is also known to naturally hybridize with other *Rubus* species such as *R. strigosus* and *R. arcticus* L (Griffiths and Ganders 1983; Viereck and Little 1972). A dense colony of salmonberry can also inhibit invasion of long-lived weedy deciduous species such as red alder, bigleaf maple and black cottonwood (Campbell and Franklin 1979).

### 1.1.6. *Rubus spectabilis* as a weedy species in conifer regeneration sites

In most north-temperate forest communities, the vast majority of plant species are not canopy trees, but rather understory herbs, shrubs and subshrubs (Thomas et al. 1999). In such forests, the fate of indigenous understory plant communities such as *R. spectabilis* is considered a function of silvicultural practices designed with the primary

intent of maximizing the value of the dominant tree crop (Thomas et al. 1999). For example, growth models for young Douglas-fir in the Pacific Northwest use percent vegetation cover as an independent variable because interspecific competition commonly limits the growth of young trees (Knowe et al. 1997).

*Rubus spectabilis* is one of the most severe competitors in many Pacific coastal forest areas and can establish dense, continuous thickets, producing pure stands of >30,000 stems/ha, >2 m tall, within 2-3 yr (Allen 1969) after a major natural disturbance (forest fires, windthrow, avalanche) or after disturbances caused by humans (logging, prescribed burning, site preparation). Invasion by *R. spectabilis* is achieved by seeding and clonal expansion from bud banks on roots and rhizomes (Tappeiner et al. 2001). Shrubs impede reforestation of logged areas by competing with planted young conifer seedlings for nutrients, moisture, space and light. Several communities of *R. spectabilis* have been found to maintain 80-100% crown closure over areas of 0.5 ha (Tappeiner et al. 1991) and can effectively reduce available light for planted young conifer seedlings. *Rubus spectabilis* is a major competitor of young conifers, such as *Tsuga heterophylla* (Raf.) Sarg., *Pseudotsuga menziesii* (Mirb.) Franco and *Picea sitchensis* (Bong.) Carr. (Barber 1976; Newton and White 1983) on moist, productive sites in most of coastal B.C. Newton and White (1983) found that 11 species of conifers overtopped by *R. spectabilis* required an additional 4.1 yr to reach survival height and further studies showed that conifers less than 60 cm tall were often killed by 4-yr old *R. spectabilis* (Newton et al. 1993). Douglas-fir has been suggested to be more susceptible to salmonberry competition than species such as western hemlock that have greater shade tolerance (Barber 1976) but other studies have shown conflicting results (Newton and White 1983).

Dense cover of *R. spectabilis* can substantially inhibit regeneration of trees through shading and smothering with mats of leaf litter.

### 1.1.7. Management

*Rubus spectabilis* can resprout rapidly after disturbance and also spread laterally by rhizome extension (Tappeiner et al. 1991). The most common way of managing *R. spectabilis* is through chemical herbicides and human manipulations. Chemical herbicide applications offer varying levels of control. Typically, herbicides cause extensive foliar damage but are often followed by resprouting because root systems remain unaffected (Oleskevich 1996). Examples of herbicides used for *R. spectabilis* control are glyphosate, hexazinone, sulfometron and triclopyr (Oleskevich 1996). Late summer and fall application, i.e., late August-early September, of glyphosate has been found to cause moderate to severe injury of *R. spectabilis* (Haeussler and Coates 1986). July-September applications of 1.4 to 2 kg a.i./ha gave good control (Newton et al. 1986; William 1994) and shrubs attained only half as much cover after spraying compared to burning (Knowe et al. 1997). Hexazinone use, broadcast spraying in March-April of sulfometron (D'Anjou 1990), and spot spraying at 0.6 kg a.i./ha or aerial spraying at 0.03 kg a.i./100 L/ha of metsulfuron have provided adequate to good control of *R. spectabilis* in site preparation practices (D'Anjou 1990; Cole et al. 1988; William 1994). Other herbicides known to provide good control of *R. spectabilis* are picloram/2,4-D combination (0.25 g a.i. + 0.9 kg a.i./ha) in the Pacific NW region (Conrad and Emmingham 1984) and triclopyr ester application in early summer at 2.9 kg a.i./ha (D'Anjou 1990).

Manual cutting or brushing is another conventional method used for controlling *R. spectabilis*. Manual cutting stimulates resprouting and regrowth of 60-90% of pre-

treatment height within one year of manual cutting (Hart and Comeau 1992), sometimes re-growing by as much as 1 m in height 3 months after cutting to ground level (Haeussler and Coates 1986). A recommended cutting in June-July has been found to temporarily reduce *R. spectabilis* but it often recovers after 9 months (Zasada et al. 1994).

In addition to herbicides and manual cutting, a variety of human manipulations have been prescribed for reduction of *R. spectabilis* cover, including site preparation such as scarification, thinning and burning. However, these methods have been found to stimulate germination and prolific resprouting, allowing stand recovery to pre-treatment levels or greater within 1-3 yr (Knowe et al. 1997). Scarification stimulates germination of buried *R. spectabilis* seeds. Scarification of clearcut areas in the Coastal Western Hemlock (CWH) zone near Vancouver Island resulted in a rapid increase in salmonberry cover over prelogging conditions and scarification was only effective if roots and rhizomes were completely removed (Haeussler and Coates 1986). Mechanical site preparation can fragment roots and result in increased stem density. It has also been found that mechanically-damaged salmonberry stems can be more resistant to glyphosate than undamaged stems (Kelpsas 1978).

Prescribed burning had some positive short term effects by delaying maximum *R. spectabilis* cover development from 3-4 yr to 4-5 yr (Knowe et al. 1997); however, long-term observations showed that by the eleventh to sixteenth season there was no difference in relative abundance of salmonberry on burned and unburned sites (Haeussler et al. 1990). In addition, burning can also cause significant site degradation (Haeussler and Coates 1986) and is not considered to be an ecologically friendly method of vegetation management in forests as well as being ineffective. Complete *R. spectabilis* cover

development is often achieved after 3-4 yr in unburned years and about a year later in burned areas (Knowe et al. 1997).

Alternative methods have also been tested to control *R. spectabilis*, such as fertilization treatments and sheep grazing. Fertilization treatments were also found ineffective and caused an increase in *R. spectabilis* cover (Thomas et al. 1999) and height and stimulation of sprouting (Jobidon 1993; Lawson and Waister 1972). Sheep grazing trials showed no decrease in the net annual growth (Sharrow et al. 1989) even though *R. spectabilis* is found to be palatable for sheep. Biological control using pathogenic fungi has been used by Oleskevich et al. (1998) and was found to be moderately effective on *R. spectabilis* under greenhouse conditions. However, the potential biological control candidate, *Fusarium avenaceum*, produced a potent mycotoxin (Oleskevich et al. 1998).

### 1.1.8. Fungal pathogens that infect *R. spectabilis*

The shrub is attacked by many plant pathogens such as fungi, bacteria and viruses, some of which can be considered for development as biological control agents. Fungal diseases can show visible symptoms such as necrosis, blight, or chlorosis, while viral diseases or latent infections can go undetected for some time. Although found with few disease symptoms in nature, *R. spectabilis* act as a reservoir for plant pathogenic microorganisms, including several fungal parasites (Fernando et al. 1999; Farr et al. 1989; Ginns 1986). Wall and Shamoun (1990a) found *Septoria rubi* West. to be the most common leaf spot pathogen of *R. spectabilis* with symptoms appearing in early June and persisting throughout the summer. Another common leaf spot pathogen in *R. spectabilis* is *Phomopsis* spp. frequently present as foliar endophytes (Shamoun and Sieber 2000). Table 1.1 summarizes the organisms reported on *R. spectabilis* in North America.

**Table 1.1.** Mycobiota of *R. spectabilis* in North America.

| Organism | Reference | Geographical location |
|---|---|---|
| *Acremonium* sp. | Shamoun and Sieber 2000 | BC |
| *Aleurobotrys botryosus* | Farr et al. 1989 | CA |
| *Aposphaeria* sp. | Shamoun and Sieber 2000 | BC |
| *Botryobasidium danicum* | Fernando et al. 1999 | BC |
| *Botryobasidium vagum* | Fernando et al. 1999 | BC |
| *Botrytis cinerea* | Farr et al. 1989 | AK |
| *Coniochaeta velutina* | Shamoun and Sieber 2000 | BC |
| *Dacrymyces cf. capitatus* | Fernando et al. 1999 | BC |
| *D. deliquescens* var. *deliquescens* | Fernando et al. 1999 | BC |
| *D. deliquescens* var. *ellisii* | Fernando et al. 1999 | BC |
| *Dichostereum pallescens* | Farr et al. 1989 | CA |
| *Geniculosporium* sp. | Shamoun and Sieber 2000 | BC |
| *Gnomoniella rubicola* | Shamoun and Sieber 2000 | BC |
| *Grandina breviseta* | Fernando et al. 1999 | BC |
| *Hymenochaete tabacina* | Fernando et al. 1999 | BC |
| *Hyphoderma pallidum* | Fernando et al. 1999; Ginns 1986 | BC |
| *Lamproderma robustum* | Fernando et al. 1999 | BC |
| *Lachnum crystalligerum* | Farr et al. 1989 | MT |
| *Marasmiellus candidus* | Fernando et al. 1999 | BC |
| *Melanconis* sp. | Shamoun and Sieber 2000 | BC |
| *Mollisia* cf. *clavata* | Shamoun and Sieber 2000 | BC |
| *Mycosphaerella rubi* | Fernando et al. 1999; Ginns 1986 | BC |
| *Nectria cinnabarina* | Farr et al. 1989 | AK |
| *Nidula candida* | Farr et al. 1989; Fernando et al. 1999; Ginns 1986 | AK, BC |
| *Nodulisporium* sp. | Shamoun and Sieber 2000 | BC |
| *Omphalina ericetorum* | Ginns 1986 | BC |
| *Peniophora cinerea* | Fernando et al. 1999; Ginns 1986 | BC |
| *Peronospora rubi* | Fernando et al. 1999; Ginns 1986 | BC |
| *Pezicula rubi* | Farr et al. 1989 | CA |
| *Phellinus ferreus* | Fernando et al. 1999; Ginns 1986 | BC |
| *Phomopsis* sp. 1, 2, 3, 4, 5 | Shamoun and Sieber 2000 | BC |
| *Polyporus badius* | Fernando et al. 1999; Ginns 1986 | BC |
| *Polyporus varius* | Farr et al. 1989 | AK |
| *Phytophthora ramorum* | Rizzo and Garbelotto 2003 | OR |
| *Pyrenopeziza rubi* | Farr et al. 1989 | WA |
| *Dasyscyphus sp.* | Fernando et al. 1999 | BC |
| *Tremella mesenterica* | Fernando et al. 1999 | BC |
| *Septoria rubi* | Farr et al. 1989; Ginns 1986 | AK, BC, CA, WA |
| *Sphaerotheca macularis* | Farr et al. 1989; Fernando et al. 1999; Ginns 1986 | AK, BC, CA, OR, WA |
| *Sphaerulina rubi* | Farr et al. 1989 | ID, MT, WA |
| *Valsa* sp. | Ginns 1986 | BC |
| *Valsa ceratosperma* | Fernando et al. 1999; Ginns 1986 | BC |

## 1.2. Biological control of forest weeds with plant pathogens

### 1.2.1. Introduction

Biological control of weeds with plant pathogens has been suggested as one of several possible means of controlling the weeds in forestry (Shamoun 2000; Wall and Shamoun 1990) and agriculture (TeBeest et al. 1992). It exploits living organisms to suppress or reduce pest populations to acceptable levels (Mortenson 1998) to where they no longer present an economic or environmental problem (Evans et al. 2001). There are several advantages to using biological control, including host specificity and biodegradability. Since biological control agents are living organisms, they can be potentially cost-effective because of their potential sustainability. Since multiple genes are involved in pathogenesis, there is a lower probability of developing resistance (Holdenrieder and Greig 1998; Wilson 1969) compared to repeated use of chemical herbicides. Disadvantages of biological control agents include limited shelf life, possible broad-spectrum target, and sensitivity to environmental conditions (Holdenrieder and Greig 1998). From a development point of view, because of specificity of biological control agents the market potential may be limited, thereby limiting profit activity required to offset development and registration costs (Mathre et al. 1999).

There are two main strategies for biological control, namely, classical (inoculative) and inundative. A third strategy, augmentative biological control, is a variant of the inundative strategy. Briefly, augmentative biological control does not require the mass-production of inoculum, requiring only a few grams per hectare to effectively control weed populations throughout the growing season (Charudattan 1991). Weed control is accomplished through an increase of disease, by way of many disease

cycles, with heavy reliance on secondary infections and dispersal of pathogens (Charudattan 1982; TeBeest 1996a).

The concept of using biological control for control of *R. spectabilis* in conifer regeneration sites is discussed below.

### 1.2.2. Classical strategy

Classical biological control involves the transfer and release of natural enemies of a pest from its native range (the center of origin or co-evolution) to another area (the target or exotic area) where natural enemies are absent, resulting in a self-sustaining balanced system in which the target pest (or weed in this case) is maintained at a non-damaging level (Evans et al. 2001). Host specificity is extremely important in classical biological control since it involves the introduction of an exotic pathogen to a target site. Classical biological control agents must cause severe damage to the target organism without damaging other organisms in the area of introduction; therefore, a thorough host range test under greenhouse conditions must be conducted to ensure the safety of native, non-target species (Mortensen 1998). Often, obligate parasites such as rusts and smuts, and insects are used for this strategy. Obligate parasites have a narrow host range. Several fungal pathogens have been introduced as classical biological control agents of exotic weeds (Evans et al. 2001), achieving varying success rates.

### 1.2.3. Inundative strategy

Inundative biological control focuses on the utilization of native fungi (mycoherbicides) or other organisms that are well adapted to the local environment as biological control agents. Inundative biological control involves the application of large doses of inoculum (Burge 1988) and often uses facultative pathogens with a very narrow

host range. This approach overcomes many of the barriers of classical biological control, such as spatial, temporal and environmental constraints. The advantage of inundative biological control over classical biological control is that indigenous fungi are subject to natural controls and will neither persist in the environment at greater than endemic levels nor spread appreciably from the treated site (Wall et al. 1992). It is self-sustaining, and long-term survival of the agent is not as important because inundative biological control agents are typically re-applied in subsequent years after initial treatment (Hintz et al. 2001). Release during the initial treatment often utilizes a large concentration of inoculum in order to overcome the natural constraints that limit epidemic development under natural conditions (Mortensen 1998). The inundative biological control agent is released by a single, timely application of inoculum at a climatically suitable time that results in infection, development of disease and eventual control or death of the specific host.

There are three key stages during the development of an inundative biological control agent: (1) discovery; (2) development; and (3) deployment.

**1.2.3.1. Discovery**

Once a target weed has been chosen, a survey of naturally occurring pathogens is conducted by collecting naturally-infected plant tissues showing disease symptoms. Searching for pathogens that occur naturally in the area infested by the weed ensures that the agent chosen will be capable of causing disease in that environment (Charudattan 1991). Under laboratory conditions, pathogens are isolated from the host, cultured and identified. Fungi collected and isolated during the survey should be identified as far as possible in order to discard non-pathogenic fungi. The fungi are tested for pathogenicity.

Pathogenicity experiments can be performed *in vitro* or under greenhouse conditions and usually involve growing the fungi to be tested in culture, harvesting their spores and applying them at a fixed concentration on the target species (Johnston and Parkes 1994). To prove that the potential biological control agent is the causal agent of the disease, Koch's postulates (Manion 1981) must be fulfilled.

#### 1.2.3.2. Development

During the development stage, literature searches and pathogenicity trials should reveal which are the most destructive pathogens among those collected during the survey (Evans et al. 2001). Pathogens capable of killing the weed have the greatest potential for inundative biological control, but highly destructive (although non-lethal) species should also be considered (Charudattan 1991). Once the most appropriate fungus has been selected, optimum conditions for inoculum production, infection and disease development, host range, stability and shelf life of inoculum and pathogen efficacy should be determined. Fungal pathogens and other organisms considered for development as a potential biological control agent must possess certain characteristics. The pathogen must be host-specific, easy to produce in the laboratory, with high virulence and ability to survive in the field.

#### 1.2.3.3. Deployment

During the deployment stage, steps towards subsequent product commercialization are considered and pursued (Watson and Wall 1995). Often the most appropriate process to commercialize a biological control agent will vary according to the pathogen and weed concerned, and the countries and companies involved (Evans et al. 2001). There are four main steps in commercialization: (1) product protection by

obtaining patents; (2) product development, i.e., mass production and formulation testing; (3) regulations and registration, which deals with concerns such as agent specification, food and feed residue, environmental fate and toxicology and efficacy; and (4) marketing and sales, which involves educating potential end users and seeking new commercial partners (Evans et al. 2001).

**1.2.3.4. Examples of inundative biological control agents for control of *R. spectabilis* and other forest weeds**

Several inundative biological control agents have been commercialized for weed management in agriculture and forestry as an alternative to chemical herbicides. In conifer regeneration sites, several hardwood species such as red alder (*Alnus rubra*), big leaf maple (*Acer macrophyllum* Pursh.), and shrubs such as thimbleberry (*Rubus parviflorus*), wild red raspberry (*R. strigosus*), salmonberry (*R. spectabilis*) and salal (*Gaultheria shallon*) are considered weedy because they compete for light, water and nutrients with planted young conifer seedlings.

Examples of inundative biological control agents developed for controlling weedy hardwood species in forestry include *Chondrostereum purpureum* (Pers.:Fr) Pouzar in the Netherlands and Canada and *Cylindrobasidium laeveae* (Pers.:Fr.) Chamuris in South Africa. *C. purpureum* is a wood-inhabiting basidiomycete fungus that grows saprobically in logs and stumps of numerous deciduous trees (Evans et al. 2001). It is able to rapidly colonize the cambium and sapwood of inoculated trees (Wall 1991) and causes a silver leaf symptom (separation of leaf layers) in Rosaceous plants (Evans et al. 2001). In Europe, *C. purpureum* is registered as BioChon™ by Koppert Biological Systems in the Netherlands for control of American black cherry (*Prunus serotina* Erhr.) (Evans et al.

2001). In North America, *C. purpureum* is marketed as Myco-Tech™ in Quebec and is being currently registered as Chontrol™ in western Canada for use as a biological control agent of unwanted tree species in utility rights-of-way sector (Evans et al. 2001). Recently, there have been plans to use native isolates of *C. purpureum* as a potential biological control agent for scotch broom and gorse in New Zealand (Evans et al. 2001). The second registered inundative biological control product is Stumpout™, which is a formulated product of the white-rot fungus *Cylindrobasidium laveae* (Pers.:Fr.) Chamuris (Evans et al. 2001; Lennox et al. 2000). Stumpout™ is registered in South Africa for control of acacia spp. (Lennox et al. 2000). In addition to weedy hardwood species, several forest shrubs are considered weedy and inundative biological control strategies have been applied on these competitors of forest regeneration. This includes *Valdensinia heterodoxa* and *Phoma exigua* for control of *G. shallon* (Shamoun et al. 2000; Vogelgsang and Shamoun 2002; Zhao and Shamoun 2005), *Colletotrichum dematium* (Pers.) Grove for control of fireweed (*Chamerion angustifolium* L. ssp. *angustifolium*) (Winder and Watson 1994) and *Fusarium avenaceum* (Fr.) Sacc. [syn. *F. roseum* Lk. (Synd. & Hans)], *C. dematium* and *Phomopsis* sp. for control of *R. strigosus*, *R. parviflorus* and *R. spectabilis* (Oleskevich et al. 1998).

Previous studies on biological control of *R. spectabilis* and other *Rubus* species worldwide have shown the potential for use of fungi as biological control agents in suppressing plant growth. Two rust fungi, *Phragmidium violaceum* (Schulz) Winter and *Kuehneola uredinis* (Lk.) Arth., were used to control European blackberry in Australia (Bruzzese 1995; Bruzzese and Hasan 1986a, 1986b) and to control native and non-native *Rubus* species in Hawaii (Gardner 1983). In addition to *F. avenaceum, C. dematium* and

*Phomopsis* sp., other indigenous fungal pathogens showing potential as biological control agents for *Rubus* spp. include *Septoria* rubi West., *Cylindrocarpon destructans* (Zinf.) Schölten (Wall and Shamoun 1990), *Hainesia lythri* (Desm.) Höhnel (Shamoun and Callan 1992) and *Didymella applanata* (Niessl) Sacc. (Thibault 1989).

### 1.2.4. Regulation

Commercial use of biological control agents as bioherbicides requires registration in Canada, as well as in the United States, just like any chemical products proposed for commercial use as pesticides or herbicides. In Canada, all applications for registration must be made to the Submission Management and Information Division, Pest Management Regulatory Agency (PMRA), Health Canada. Regulation of registered products for pest control or pesticides will be legislated under the Pest Control Products Act (PCPA), which received Royal Assent on December 12, 2002 and will be administered by the Pest Management Regulatory Agency. Regulatory guideline proposals for microbial pest control agents with components such as efficacy and benefits, environmental toxicology and fate, food, feed residue and metabolism studies, human health and safety testing and characterization and specification have been published (Anonymous 2001). However, the new PCPA is not yet enforced as of 2005 and the enforcement date is still yet to be determined. In the U.S., the Environmental Protection Agency (EPA) regulates pesticides under two laws, namely, the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetics Act (FFDCA), as amended by the Food Quality Protection Act (FQPA).

### 1.2.5. Host range

Host range generally refers to the set of species on which a biological control organism can feed and develop. The safe and effective use of biological control agents require assessment of the biological control organism's ability to harm non-target organisms, to survive, reproduce, disperse, and evolve. Ideally, biological control represents a pest control technology that is effective, safe on the environment, and largely self-sustaining, with minimal need for repeated and costly pest control interventions. However, some of the characteristics that make biological control organisms effective in controlling pests also make them potentially dangerous invaders. Therefore, the host range of a potential biological control agent is tested to determine its host specificity and to determine its safety as well as potential risks on non-target plant species following release. Host specificity tests typically measure the potential of the control organism to complete its life cycle on the target organism and also on the non-target organisms that it consumes (eats, parasitizes, or infects). The biological control agent can harm a non-target organism in several ways – from a direct trophic interaction that arises when the biological control organism consumes a non-target organism; or to a direct interference competition; or to indirect interactions that can arise when the biological control organism and the non-target organism interact via intermediate species such as a shared natural enemy or a shared host.

Because it would be impossible to test all plant species, techniques have been utilized for testing host specificity. Biologically-relevant testing methods (Harris and Zwolfer 1968) are based on investigations of the physiological, morphological, phenological, entomological, and chemical bases of host restriction, combined with

testing plants that have any of several critical properties: they are related to the target host, they are host plants of related insects, the agent has occasionally been recorded on them, they have characteristics in common with the target weed. The centrifugal/phylogenetic testing procedure (Wapshere 1974) is an example of a biologically relevant method. This method involves testing plants of increasingly distant relationship to the host until the host range is circumscribed with the target host at the center. A margin of safety is added by testing all related plants of economic (e.g., crops and horticultural plants) and ecological (e.g., threatened and endangered plants in the native flora) value that could be considered "at risk" from the biological control agent. Plants that are considered as potentially at risk include: those related to the weed, those without adequate prior exposure to the agent for ecological or geographical reasons, those for which little is known about the insects or fungi attacking them, those with secondary chemicals or morphological features similar to those of the target host weed, and those attacked by close relatives of the biological control organism.

Another method relies on the relatedness procedure or variants thereof. The relatedness procedure is another biologically-relevant method but instead of being additive like the centrifugal/phylogenetic procedure, it is a subtractive procedure that involves ordering plants to be tested based on their relationship to the target organism. The procedure is weighted to favor those potential hosts that are most closely related to the target organism but is designed to include representatives from all levels of relationship to the target. The relatedness procedure provides a more representative sample of the entire flora, decreasing the likelihood that suitable hosts unrelated to the target will be missed. It has been applied successfully in the host specificity of

*Uromysces heliotropii*, a fungal agent for the biological control of common heliotrope (*Heliotropium europaeum*) in Australia (Hasan et al. 1992). The test was conducted by inoculating 96 plants important to the Australasian region followed by both microscopic and macroscopic observations that examined reactions in host and non-target host plants. When testing for non-target host range, it is also important to detect latent colonization and infection by pathogens. This was an important factor tested by Mortensen and Makowski (1997) during the development of *Colletotrichum gloeosporioides* f. sp. *malvae* as a biological control agent.

## 1.3. Evaluation of *Didymella applanata* (anamorph *Phoma argillacea*) as a potential biological control agent for *R. spectabilis*

### 1.3.1. Life cycle and biology

*Didymella applanata* is a parasitic ascomycetous fungus that causes spur blight or cane blight and irregular leaf necroses mainly on cultivated raspberries in North America, as well as other species in the genus *Rubus*, including blackberry, loganberry and thimbleberry (Table 1.2).

It has been known for approximately 80 years that *D. applanata* has a *Phoma* sp. anamorph (Koch 1931). The anamorphic state of *D. applanata* was recently named as *Phoma argillacea* (de Gruyter et al. 2002). *P. argillacea* produces ostiolate pycnidia (40-320 μm diam), and hyaline aseptate conidia, 4-7.5 x 2-4 μm in size *in vitro* (de Gruyter etal. 2002). *Phoma argillacea* has also been noted to produce larger 1-septate conidia (up to 13 x 4 μm) *in vitro*. Pycnidia *in vivo* are often found scattered on stem lesions throughout the summer and autumn, as well as on necrotic lesions on leaves (de Gruyter et al. 2002). *Phoma argillacea* has a slow growth rate on oat agar (OA) (<35 mm after 7

**Table 1.2.** Host species of *D. applanata* in Canada*.

| Host species | Common Name | Family | Province Isolated From |
|---|---|---|---|
| *Rubus idaeus L.* | Red raspberry | Rosaceae | British Columbia<br>Alberta<br>Saskatchewan<br>New Brunswick<br>Nova Scotia<br>Ontario<br>Quebec |
| *R. idaeus* var. *strigosus* | Red raspberry | Rosaceae | British Columbia |
| *R. ursinus* var. *loganobaccus* | Loganberry | Rosaceae | British Columbia |
| *R. parviflorus* | Thimbleberry | Rosaceae | British Columbia |

*All data is from Ginns (1986) and Fernando et al. (1999), except for information on *R. parviflorus* (Shamoun and Sieber 2000).

days) and it does not produce crystals or chlamydospores on agar medium (de Gruyter et al. 2002). *Phoma argillacea* produces a negative response for the presence of antibiotic "E" using the diagnostic NaOH spot test (de Gruyter et al. 2002).

The teleomorphic stage of *D. applanata* is morphologically characterized by having 4- to 8-septate two-celled ascospores within a bitunicate ascus. The asci are borne on discrete ascomata immersed on the host tissue. The ascospores are hyaline, smooth, ellipsoidal and constricted at the septum. Phylogenetic analysis of combined ITS1, 5.8S and ITS2 sequences (490 nt) showed that nucleotide sequences of several *D. applanata* isolates collected from two different countries, Sweden and Netherlands, were 99.8-100% identical forming a monophyletic clade (Lindqvist-Kreuze et al. 2003). *Didymella applanata* was placed in the *Phoma-Didymella* clade (Venturiaceae) as revealed in a phylogenetic study of *Phoma* spp. using ITS1 along with several *Phoma* species such as *P. macrostoma, P. nebulosa* and *P. sorghina* (Reddy et al. 1998).

### 1.3.2. Taxonomy of *P. argillacea*

*Didymella applanata* is an ascomycetous fungus that produces bitunicate asci and it belongs to the order Dothideales. *Didymella applanata* was first referred by Niessl. in 1875 in Europe as *Didymosphaeria applanata* (Koch 1931). Saccardo later transferred it to *Didymella* in 1892 (Koch 1931). It was subsequently described in North America in 1894 as the causal fungus of spur blight on *Rubus* species specifically *Rubus idaeus* (red raspberry).

As previously mentioned, classification of ascomycetes is based on the type of ascomata (apothecia, cleistothecia or perithecia), which are multicellular structures that act as platforms from which the spores are launched, as well as the type of asci. The order

Dothideales is composed of all non-lichenized ascomycetes with bitunicate asci (von Arx 1987). Within this order there are 2 suborders (von Arx 1987) and it contains 50 families, 650 genera with 6300 species described (Kendrick 2000), which includes many fungi parasitic on cultivated plants which causes leaf spot, necrosis, scab or dieback. The genus *Didymella* is a member of the Pleosporaceae Winter sensu lato (Corlett 1981). *Didymella* is characterized by separate immersed pseudothecia, with clavate to cylindrical, parallel, eight-spored asci arising from a broad hymenium among pseudoparaphyses and by hyaline one-septate ascospores (Corlett 1981). *Didymella* includes many parasitic or saprophytic fungi, causing necrosis, stem- and fruit-rot, blight and leaf spot on a number of plant species (Table 1.3). Known anamorphs of *Didymella* are *Ascochyta* (occasionally treated as *Diplodina*) and *Phoma* (von Arx 1987). *Didymella* was delimited into 19 species by Corbaz in 1957 (von Arx 1987) and 9 species were described by von Arx (1987). Misidentification of *Didymella* as *Mycosphaerella* and vice versa are known to occur often because of morphological similarity, except *Mycosphaerella* has fasiculate asci without paraphysis, has smaller ascomata and it does not have a *Phoma* or *Ascochyta* anamorphs.

The anamorphic state of *D. applanata* was recently named as *Phoma argillacea* (de Gruyter et al. 2002). Generally, the genus *Phoma* is delimited from other coelomycetes by having hyaline conidia, which remains one-celled *in vitro*, within pycnidial conidiomata, and conidial septation is often a secondary process. The genus *Phoma* was subdivided into 9 sections by Boerema (1997) wherein *P. argillacea* was placed in the section Phyllistictoides.

**Table 1.3.** Host range of 9 *Didymella* species*.

| Fungi | Host species | Tissue Isolated From |
|---|---|---|
| *Didymella applanata* | *Rubus idaeus, R. arcticus* | Spur blight, leaf lesions |
| *D. bryoniae* | Bryoniae, Citrullus, Cucumis, Cucurbita | ** |
| *D. cannabis* | Cannabis | Stem decay |
| *D. exitialis* | Hordecum, Triticum, Poaceae | Culms, leaves, inflorescence |
| *D. ligulicola* | Chrysanthemum, Pyrethrum | |
| *D. lycopersici* | *Solanum lycopersicum* | Stem rot/decay |
| *D. pinodes* | *Pisum sativum*, other Fabaceae | Stem rot/decay |
| *D. rabiaei* | Cicer | Stem rot, leaf decay |
| *D. sorghina* | Sorghum, other Poaeceae | Stem rot, leaf decay |
| *D. zeae-maydis* | Maize, other Poaceae | Leaf spots |

* All data were obtained from von Arx (1987), plus Lindqvist-Kreuze et al. (2003) for *D. applanata* on *R. arcticus*. Consult von Arx (1987) and Lindqvist-Kreuze et al. (2003) for authority.

** Not indicated in literature.

There are approximately 2000 putative *Phoma* species (Sutton 1980), many of these are synonyms or improperly placed within this genus. Because of this, there are around 200 accepted *Phoma* species to date. The genus *Phoma* contains several important plant pathogens, including *P. exigua*. *Phoma exigua* can be easily identified from other *Phoma* species using a diagnostic sodium hydroxide (NaOH) spot test for the presence of a colourless metabolite antibiotic "E". A drop of NaOH causes colonies on malt agar (MA) to initially turn blue-green and subsequently turning brownish-red. Antibiotic "E" is believed to be stimulated by light and has bactericidal and fungicidal properties.

Although identification of fungi has vastly improved through the use of stable cultural characteristics, irregularities such as failure to sporulate and inconsistencies in septation can occur in *Phoma* and *Didymella*. In addition, *Phoma* contains anamorphs of multiple teleomorphic genera as previously mentioned, creating a systematic problem. More recently, DNA markers (ribosomal and nuclear) are being used to identify *Phoma* and other fungal species. Sequence data generally support traditional fungal taxonomy and has for instance been able to group ascomycetes and basidiomycetes into discrete monophyletic groups (Berbee and Taylor 1993), as well as resolve relationships indeterminable based on morphology. The ribosomal DNA internal transcribed spacers, ITS1 and ITS2, have been used to identify several fungi at the genus level, while nuclear DNA markers such as RAPDs and AFLPs are being used to establish relationships at or below the species level (Abeln et al. 2002; Kothera et al. 2003). AFLP markers were found very useful in showing differences among different varieties of *P. exigua*, while ITS markers were only able to differentiate among different species of *Phoma* (Abeln et

al. 2002). Results of RAPD analysis also supported the distinction between pathogenic and non-pathogenic (or weakly virulent) isolates of *D. bryoniae* and *Phoma* spp., respectively (Keinath et al. 1994 and1995). ITS markers have proven useful in linking anamorphs with their teleomorphs. For example, recent ITS sequencing of *Epicoccum nigrum* and *Phoma epicoccina* has shown that these two organisms are the same biological species (Arenal et al. 2000). Based on these recent studies, genetic markers can be used as a powerful tool that can support phenotypic or morphological differences among fungal species.

*Phoma argillacea* has been recently linked to its *D. applanata* teleomorph using both cultural and molecular characteristics (Lindqvist-Kreuze et al. 2003). Culturally, both species showed similar growth rate on OA and similar conidial dimensions. Phylogenetic analysis showed a very similar nucleotide sequence between *D. applanata* and *Phoma* sp. isolated from *Rubus*, with only 1.7% sequence divergence constituting two point mutations in the ITS2 region, C→T at position 352 and A→G at position 365 (Lindqvist-Kreuze et al. 2003).

### 1.3.3. *Phoma argillacea* host range

*Didymella applanata* is widely distributed in North America, Australia and Europe. In the US and Canada, *D. applanata* has predominantly been isolated from raspberry in 7 out of 9 Canadian provinces (Table 1.2) and is commonly found in Washington, Oregon and Idaho in the US (Ginns 1986). In British Columbia, *D. applanata* has been isolated from *R. idaeus, R. idaeus* var. *strigosus* and *R. loganobaccus* and as an endophyte on *R. parviflorus* (Table 1.2). In Europe, *D. applanata* has been

isolated from *R. arcticus* nothossp. *stellarcticus* (arctic bramble) in Sweden in addition to *R. idaeus*.

### 1.3.4. Fungal mode of infection

*Didymella applanata* attacks the canes (or stems), leaves and buds of *R. idaeus* and can cause extensive wilting on cultivated *R. arcticus*. Infection by *D. applanata* results in the destruction of some or all of the fruiting branches or spurs that grow towards the basal parts of the canes (Lousberg et al. 1973). In addition, infection of leaves of young canes results in impaired vitality of the leaves, with young buds becoming shrunken and shrivelled (Lousberg et al. 1973). Buds are seldom killed because they are not invaded by the fungus but are often weakened and reduced in size so that they fail to develop when in competition with uninfected buds (Jennings 1988) and the plant becomes more prone to frost damage. Some cultivars of *R. idaeus* have been found to be more resistant to *D. applanata*, with cane morphology as the primary contributing factor to resistance, i.e., hairy canes tend to be less affected and more resistant to mycelial inoculation of wounds (Jennings 1988).

Spur blight is a disease specific to *Rubus,* with *R. idaeus* being most seriously affected. Infections on leaves of primocanes are initiated by the appearance of small brown lesions at leaf margins and near the large leaf veins, often separated from green tissue by a yellow band, which advance inward toward the midvein (Ellis et al. 1988). The leaves are the main infection sites and disease eventually spreads to the petiole and into the node. Histological evidence shows that the fungus freely invades the outer tissues of the cane beneath the node (Jennings 1988). It only affects the primary cortex of the cane and if lesions are scraped, healthy green vascular tissues are exposed beneath

the cork. The fungus grows basipetally within the petioles and colonizes the cortex of the cane around the buds and the undersides of the swollen leaf bases, where it kills the cells and induces a chestnut-brown lesion (Jennings 1988).

The fungus survives as mycelium, pseudothecia and pycnidia on infected canes during the winter (Ellis et al. 1988). Under wet conditions during the summer, it produces ascospores and conidia, which infect the leaves of primocanes. Lesions appear in June or July and progress higher on canes. Ascospores, which are dispersed by air currents and splashing rain, are discharged in early to late Summer to young tender canes where they directly penetrate thesteles. Conidia are dispersed by splashing in July and August to other leaves and canes, with infection initiated in both wounded and unwounded tissues. Infected plants become highly susceptible as they begin to senesce when light is excluded from the cane bases (Jennings 1988). By late July or August, primocanes are covered with spur blight lesions at the nodes, which become silvery grey in the winter. These lesions contain masses of pycnidia or pseudothecia (Ellis et al. 1988)

There are two sources of inoculum, the ascospores of *D. applanata* and the conidia of *P. argillacea.* Conidia are considered to be the major source of infection. Ascospores are discharged from a bitunicate ascus and are dispersed by air currents and rain splash. Conidia are released from pycnidia and are dispersed by rain splash. Entry to the host can be either through wounded or unwounded tissues. Easy entry through wounds has been reported to be facilitated by the raspberry midge *Thomasiniana theobaldi*, which deposits its eggs under the epidermis of young raspberry stalks (Schuring and Salemink 1972). In addition, *D. applanata* produces an exocellular

phytotoxic oligosaccharide, which is able to induce similar symptoms when tested on raspberry shoots as the fungus in the plants (Lousberg et al. 1973). This toxin was further characterized by Broekhoven et al. (1975) as a large glycopeptide (molecular weight of ~63,000) possessing a molecular formula of $C_{357}H_{677}O_{296}N_{13}S)_7$ with glucose as its main constituent. This exocellular toxin has been linked with the induction of chestnut brown lesions that kill cells.

The relative virulence of a pathogen affects successful infection of its host. Two pathotypes of *D. applanata* that differ in their ability to infect the cultivar Newburgh have been described in eastern Canada (Ellis et al. 1988). Red raspberry is known to induce increased peroxidase activity as a defense response to *D. applanata* (Kowzlowska et al. 2001). In this study, increased peroxidase activity was dependent on the zone of cane growth related to the degree of lignification and thickness of the cortex layer (Kowzlowska et al. 2001). However, the spread of disease has been related to periods of plant susceptibility during its growth phase (Jennings 1988). Susceptibility of canes is greatly increased by a period of relatively high temperature before attack, i.e., warmer spring and autumn conditions (Williamson and Pepin 1987). This is probably due to increased inoculum potential resulting from the dispersal of conidia.

## 1.4. Objectives of research

The main objectives of this study were to: 1) identify and discover naturally occurring fungi as potential biological control agents for *R. spectabilis*; 2) determine the environmental requirements of the potential biological control candidate(s) to infect *R. spectabilis*; and 3) determine the genetic diversity of the target host *R. spectabilis* in its natural range.

# CHAPTER 2. OCCURRENCE OF *PHOMA ARGILLACEA* ON *RUBUS SPECTABILIS* IN BRITISH COLUMBIA AND AN EVALUATION OF ITS POTENTIAL AS A FOREST WEED BIOLOGICAL CONTROL AGENT

## 2.1. Introduction

*Rubus spectabilis* Pursh. (salmonberry) is a native perennial deciduous shrub in North America (Oleskevich et al. 1996) and is considered to be one of the top 20 forest weeds in Canada (Wall et al. 1992). Salmonberry negatively impacts successful establishment and growth of young conifers, such as Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and sitka spruce (*Picea sitchensis* (Bong.) Carr.) (Barber 1976; Newton and White 1983). Once established, control of salmonberry is difficult since it requires a reduction of the bud bank in the ramets and rhizomes (Tappeiner et al. 1991). Manual cutting, often with chainsaws, results in rapid replacement of cut stems (Zasada et al. 1992). Generally, the most effective and economical method for suppression of forest weeds is by manual cutting followed by application of chemical herbicides. However, persistence of herbicides in the environment, water contamination following aerial spraying and runoff from land treated with herbicides (Selim et al. 1989) have prompted the need for alternative, more environmentally-friendly and socially-acceptable methods for controlling forest weeds. Biological control of forest weeds using inundative applications of fungal pathogens may provide such an alternative (Evans et al. 2001; Shamoun 2000; Wall et al. 1992).

Previous studies on biological control of salmonberry and other *Rubus* species worldwide have shown the potential use of fungi as biological control agents in

suppressing plant growth. Two rust fungi, *Phragmidium violaceum* (Schulz) Winter and *Kuehneola uredinis* (Lk.) Arth., were used to control European blackberry in Australia (Bruzzese 1995; Bruzzese and Hasan 1986a, 1986b) and to control native and non-native *Rubus* species in Hawaii (Gardner 1983). In Canada, several indigenous fungal pathogens, including *Fusarium avenaceum* (Fr.) Sacc. [syn. *F. roseum* Lk. (Synd. & Hans)] (Oleskevich et al. 1998), *Septoria* rubi West., *Cylindrocarpon destructans* (Zinf.) Schölten (Wall and Shamoun 1990), *Hainesia lythri* (Desm.) Höhnel (Shamoun and Callan 1992) and *Didymella applanata* (Niessl) Sacc. (Thibault 1989) have shown potential as biological control agents of *Rubus* species.

In this study, we investigate the potential of using naturally occurring fungi for biological control of salmonberry and describe the use of the newly discovered *Phoma argillacea* (Bres.) Aa & Boerema comb. nov. (teleomorph *Didymella applanata* (Niessl) Sacc.) as a novel candidate biological control agent for management of salmonberry.

## 2.2. Materials and methods

### 2.2.1. Pathogen survey

Naturally-infected salmonberry foliage and stems showing disease symptoms that included necrosis, shoot dieback, and stem blight, were collected during August 2000 from 16 different sites on Vancouver Island and coastal British Columbia (Table 2.1). Diseased plant tissues (ca. 0.25 $cm^2$) were excised and surface-sterilized by successive 1-min rinses in 95% ethanol, 0.525% sodium hypochlorite (w/v) and three rinses in sterile distilled water. Tissues were blotted on sterile filter paper, aseptically plated onto malt extract agar (MEA) or potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) and incubated at 20-25°C in the dark. Resulting fungal colonies were hyphal-tipped and pure

**Table 2.1.** Locations and descriptions of sites throughout Vancouver Island and coastal mainland British Columbia where diseased salmonberry was collected.

| Site | Date | Location | Latitude and longitude | General site description |
|---|---|---|---|---|
| 1 | 07/20/00 | Windsor Lake, Powell River (WL22) | 50°01'08"N 124°18'31"W | Clearcut |
| 2 | 07/20/00 | Powell River (Block 922) | 49°55'18"N 124°19'55"W | Clearcut |
| 3 | 07/21/00 | Powell River (ST41) | 49°46'05"N 124°18'08"W | Clearcut |
| 4 | 07/21/00 | Powell River (HB12) | 49°54'23"N 124°30'30"W | Clearcut |
| 5 | 07/21/00 | Powell River (HB15) | 49°53'53"N 124°30'44"W | Clearcut |
| 6 | 07/21/00 | Powell River (HB19) | 49°56'02"N 124°28'38"W | Clearcut |
| 7 | 07/21/00 | Powell River (HB7) | 49°53'20"N 124°29'49"W | Clearcut |
| 8 | 07/31/00 | Jordan River (RS8) | 48°25'43"N 124°03'17"W | Roadside |
| 9 | 07/31/00 | French Beach (RS9) | 48°23'N 123°57'W | Mature forest edge |
| 10 | 07/31/00 | Loss Creek (RS10) | 48°29'43"N 124°15'29"W | Roadside |
| 11 | 08/15/00 | Mesachie Lake (RS11) | 48°49'34"N 124°08'18"W | Along seasonal stream |
| 12 | 08/15/00 | Gordon River (RS12) | 48°49'34"N 124°23'18"W | Logging road |
| 13 | 08/15/00 | Honeymoon Bay (RS13) | 48°49'34"N 124°12'54"W | Clearcut |
| 14 | 08/23/00 | Lion's Bay, Deeks Lake Trail (RS14) | 49°32'37"N 123°14'40"W | Logging road |
| 15 | 09/13/00 | Sarita (RS15) | 48°46'08"N 125°04'32"W | Clearcut |
| 16 | 10/10/01 | Victoria | 48°27'61"N 120°23'82"W | Roadside |

cultures were stored on MEA or PDA slants at 5°C with periodic testing for viability. Fungi were identified to genus and in some cases to species using morphological characteristics (von Arx 1981; 1987; Barnett and Hunter 1998; Hanlin 1998).

### 2.2.2. *In vitro* pathogenicity tests

An *in vitro* pathogenicity test was performed on 281 fungal cultures using detached salmonberry leaves obtained from greenhouse-grown plants. Leaves were placed on moistened paper towels in plastic trays and inoculated with mycelial plugs (1-cm diam) taken from 7-10 day old colonies, with 4 replicate leaves per isolate. Leaves were incubated for 14 days in a growth chamber set at day/night temperatures of 21°C/18°C and a 12 hr photoperiod. Control leaves were inoculated with sterile PDA plugs and were incubated under identical conditions. Percent leaf area damage was assessed visually (Oleskevich et al. 1998) and a mean percent necrosis of >50% after 14 days indicated moderate to high pathogenicity of isolates. Fungi causing >50% necrosis were re-isolated in order to fulfill Koch's postulates. Following this screening, three *Phoma* isolates (PFC 3313, PFC 3707 and PFC 3714) were selected for further morphological characterization and pathogenicity tests using whole plants. Cultures were sent to Dr. R.A. Samson (Centraalbureau voor Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, The Netherlands) to obtain the species designation of the *Phoma* isolates.

### 2.2.3. Colony growth and spore germination

Optimum colony growth and spore germination of *Phoma* sp. were determined at temperatures ranging from 0°C to 35°C, in 5°C increments, in the dark. For colony growth tests, colony diameter on PDA was measured after 14 days. For spore germination tests, masses of conidia contained in pycnidia were obtained from colonies

grown on V-8 juice agar under continuous light at room temperature (20-22°C) by flooding plates with sterile distilled water and gently dislodging the conidia with a sterile glass rod. Conidial suspensions were diluted to 1 x $10^6$ spores/mL and 500 µL was spread onto 2% water agar plates and incubated in the dark. Percent germination was assessed after 24 hr from a total of 300 spores per plate at each temperature. For both tests, there were 5 replicate plates of each isolate at each temperature and the experiments were repeated once.

### 2.2.4. Greenhouse virulence tests

#### 2.2.4.1. Propagation of plants

Salmonberry plants were obtained from a single field rootstock collected in Coal Harbour, BC. After a 3-month cold stratification period at 0°C, the rootstock was initially planted in peat-perlite (1:1) medium to force root formation and flush new stems and leaves. Stem segments (10-cm-long) with 2-3 leaves were then obtained from the stock plant, dipped in 0.4% indole-3 butyric acid rooting powder and planted in peat-perlite (1:2) and placed in a mist chamber for 3-4 weeks. Healthy plants were transplanted to peat-vermiculite-sand (3:1:1) medium with a low rate of slow-release fertilizer and maintained in 10-cm diameter pots in the greenhouse for 3-4 months.

#### 2.2.4.2. Inoculum preparation

Mycelial suspensions of the 3 *Phoma* isolates (PFC 3313, PFC 3707 and PFC 3714) were prepared by inoculating 100 mL of potato dextrose broth (PDB) (Difco Laboratories, Detroit, MI) contained in a 250-mL Erlenmeyer flask with 10 mycelial plugs (1-cm diam) taken from the actively growing margin of 7-10 day old cultures. Cultures were incubated for 11 days at room temperature (20 -22°C) on a rocking shaker

(MetaBios Inc., Victoria, B.C.) at 20 rpm. The mycelium was macerated for 30 s at low speed, followed by 15 s at high speed, repeated once, in a surface-sterilized blender. Inoculum concentration was quantified by serial dilutions onto PDA and enumerating colonies after 2 days of incubation at 20-25°C in the dark. Conidia were produced as mentioned previously and the concentration was adjusted to 1 x $10^6$ spores/mL in sterile PDB. Macerated mycelia or spores were separately sprayed to runoff with a hand-held sprayer onto 5 replicate plants.

Post-inoculation conditions, namely, the effect of inoculum type, leaf wetness period and inoculum concentration on salmonberry plants were investigated using PFC 3313. In most of the greenhouse experiments, following inoculation, plants were covered with a clear plastic bag for 48 hr after which the bags were removed and the plants were maintained under greenhouse conditions (20-22°C, 55-65% RH, with a 16-hr photoperiod). Plants were rated for up to 3 weeks after inoculation and compared to control treatments sprayed with sterile PDB. Disease severity was measured as the percentage of infected leaves per plant.

**2.2.4.3. Effect of post-inoculation conditions**

The effect of mycelial inoculum on salmonberry plants was compared to spore inoculum to determine which type of inoculum causes the greatest damage on salmonberry. It was found that mycelia were more effective than spores; therefore, subsequent greenhouse experiments were conducted using mycelia as inoculum.

The effect of leaf wetness period on infection was investigated by providing the inoculated plants with 3, 6, 12, 18, 24, 36 or 48 hr of continued leaf wetness after inoculation. The clear plastic bags were removed after the respective times and the plants

were returned to an ambient greenhouse environment as described previously. After 21 days, dry-matter accumulation in the remaining living aerial parts of the plant was determined.

The effect of inoculum concentration (mycelial) on infection was investigated by diluting macerated mycelia (11-days old) to suspensions of 0.1, 0.5, 5 and 10% mycelium (v/v) with PDB and applied to plants as described above. Plants received a 48 hr period of leaf wetness.

Finally, a phytotoxicity test was conducted to determine if phytotoxic compounds were produced in culture and contributing to disease on inoculated plants. Mycelia were grown in 0.6, 1.2 and 2.4% (w/v) PDB for 11 days and the culture filtrates were filtered through cheesecloth and sprayed to run-off as before. Prior to inoculation, filtrate quality was assessed by plating a sample of the culture filtrates onto PDA to confirm complete removal of mycelia.

### 2.2.5. Effect of *P. argillacea* on non-target hosts

Seedlings (one-year-old) of three economically important conifer species, namely, Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and western red cedar (*Thuja plicata* D. Don.), were planted in 4.5-litre pots in a peat-vermiculite-sand (3:1:1) mix under greenhouse conditions. Seedlings were sprayed with *P. argillacea* (PFC 3313) mycelial suspension (1-2 x $10^5$ CFU/mL) obtained from 11-d old liquid culture. Inoculation procedures were as described previously using 10 replicate seedlings per conifer species. The seedlings were examined for necrotic symptoms over 3 weeks. Assessments of seedling tolerance to treatment applications were made according to the Expert Committees on Weeds (ECW)

Western Canada Section Rating Scale (0-100%), where >10% damage to conifer seedlings is unacceptable injury (Anonymous 1996).

Since *Didymella applanata*, the teleomorph of *P. argillacea,* is the causal agent of spur blight on cultivated red raspberries (Koch 1931), a pathogenicity test using isolate PFC 3313 was conducted to determine infection of red raspberry cultivar 'Meeker'. Mycelial suspensions (1-2 x $10^5$ CFU/mL) were prepared and sprayed onto 5 test plants as described previously.

### 2.2.6. Data analysis

In all greenhouse experiments, disease was rated over a 3-week period from replicate plants. All experiments were performed twice. The data collected were analyzed using one-way ANOVA to determine the effect of various treatments. Differences between means in all pairs were tested by the Tukey-Kramer HSD (honestly significant difference) method (Tukey 1953; Kramer 1956) at $P = 0.05$ using JMP ver. 4.1 (SAS Institute, Cary, NC, USA). All experiments were performed twice.

## 2.3. Results and Discussion

### 2.3.1. Pathogen survey

Diseased salmonberry leaves and stems collected from 16 locations on Vancouver Island and coastal mainland British Columbia (Table 2.1) yielded 330 cultures of fungi comprising 20 genera (Table 2.2). The most frequently recovered genera were *Phomopsis* and *Botrytis* from 11 and 9 different locations, respectively. Of the 330 cultures, 281 were screened for pathogenicity on detached salmonberry leaves. Seventy-six isolates caused leaf necroses ranging from >1% to 100% after 14 days (data not shown). A majority of these fungi were weak pathogens and only 19 isolates caused

**Table 2.2.** Genera of fungi recovered from diseased salmonberry tissues collected on Vancouver Island and coastal British Columbia.

| Fungal genus | Sites containing Genus * | No. of isolates recovered** | Pathogenicity range after 14 days (%) |
|---|---|---|---|
| *Botrytis* | 1, 2, 3, 4, 8, 9, 10, 12, 16 | 17 | 10-100 |
| *Chaetomium* | 8, 10 | 2 | 0 |
| *Cladosporium* | 1, 3, 5, 9, 10 | 5 | 0 |
| *Coniochaeta* | 2 | 1 | 0 |
| *Dictyosporium* | 2 | 1 | 0 |
| *Diplodina* | 1 | 1 | 0 |
| *Endothiella* | 7 | 1 | 0 |
| *Epicoccum* | 3, 7, 9, 16 | 5 | 0-40 |
| *Fusarium* | 3, 4, 10 | 5 | 10-30 |
| *Microsphaeropsis* | 1, 3, 4, 5, 6 | 7 | 0-10 |
| *Monochaetia* | 3 | 2 | 0 |
| *Nodulisporium* | 10 | 1 | 0 |
| *Penicillium* | 1, 8 | 4 | 0 |
| *Phoma* | 8, 11, 16 | 4 | 1-70 |
| *Phomopsis* | 1, 2, 3, 4, 5, 6, 8, 10, 11, 14, 16 | 24 | 0-40 |
| *Pilidium concavum* | 6 | 1 | 20 |
| *Septoria* | 1, 3, 8, 15 | 8 | 1-55 |
| *Tilaclidium* | 2 | 1 | 0 |
| *Trichoderma* | 6 | 1 | 0 |
| *Zythia* | 1 | 1 | 0 |

* Numbers refer to site locations listed in Table 2.1.

**Additional unidentified isolates were initially tested for pathogenicity, a majority of which (154/189 =81%) were found to be non-pathogenic on detached salmonberry leaves. The average pathogenicity range for the unidentified pathogenic isolates was 12.3% (n = 35).

>50% necrosis after 14 days. These included 15 *Botrytis cinerea* isolates, 3 *Phoma* isolates and 1 Septoria isolate. Botrytis cinerea was not selected for further study because of its wide host range and potential to infect young conifer seedlings and other horticulturally- important species. The slow growth rate in artificial medium of *Septoria*, difficulties in mass production of spores in culture and relatively low post-inoculation temperature requirements (Wall and Shamoun 1990) excluded it from further consideration in this study. Consequently, only the three isolates of *Phoma* (PFC 3313, PFC 3707 and PFC 3714) collected from infected foliage on Southern Vancouver Island (one isolate from site 11 and two from site 16) (Table 2.1) which caused >50% foliar necroses after 14 days were selected for further study.

### 2.3.2. Colony growth and spore germination

Of the range of temperatures tested, maximum colony growth and spore germination of the 3 isolates of *Phoma* sp. were observed at 20°C and 25°C, respectively (Figures 2.1, 2.2). The average colony growth at 20°C was 63 mm, 59.6 mm and 58.6 mm for PFC 3313, PFC 3707 and PFC 3714, respectively, and spore germination was >95% at 25°C for all three isolates. Temperatures below 5°C and above 30°C inhibited both colony growth and spore germination.

Pycnidia development was observed in culture and on necrotic lesions (Figure 2.3A). Pycnidiospores measured (6-) 8 x 2 (-2.5) μm and were hyaline and aseptate, ellipsoidal or cylindrical in shape (Figure 2.3B). Identification of *Phoma* sp. as *P. argillacea* was confirmed by Dr. R.A. Samson (Centraalbureau voor Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, The Netherlands). Cultural and morphological characters agreed with the description by de Gruyter et al. (2002) of *P. argillacea*, the anamorph of

*Didymella applanata* (Niessl) Saccardo, except that my isolates did not produce any 2-celled conidia in culture. In addition, the complete ITS sequence (ITS1-5.8SrDNA-ITS2) was found to be almost identical (99.4% homology) to the complete ITS sequence of *D. applanata* (Niessl) Saccardo available in GenBank, e.g., AJ428170, AJ428169.

### 2.3.3. Greenhouse virulence tests

All three isolates of *P. argillacea* produced significant and reproducible foliar and stem necrosis on salmonberry plants in greenhouse trials. Leaves appeared dry, curled and brown, with damage extending to branches and stems (Figure 2.3C, D), often resembling a phytotoxic effect on the plant. There was no significant difference in the mean percent necrosis among the three isolates (Table 2.3). However, macerated mycelial inoculum was found to cause significantly greater disease compared with spores, except for isolate PFC 3707 (Table 2.4). This confirms recent reports that *Phoma* mycelia are more infective than spores (Neumann and Boland 2002; Stewart-Wade and Boland 2004). Spraying of a mycelial suspension resulted in the development of foliar necroses after 48 hr, and pycnidia containing masses of conidia were observed on the leaves after 5 days (Figure 2.3A). Symptoms did not develop on control plants, indicating that PDB is a sufficient carrier to support fungal growth with no phytotoxic effect. *Phoma argillacea* was re-isolated from necrotic leaf tissues of inoculated salmonberry plants and not from control plants. Re-growth of new leaves from treated and diseased plants resumed after three weeks, often initiating from uninfected stem areas. New stems were also produced in some treated plants and arose from below-ground rhizomes.

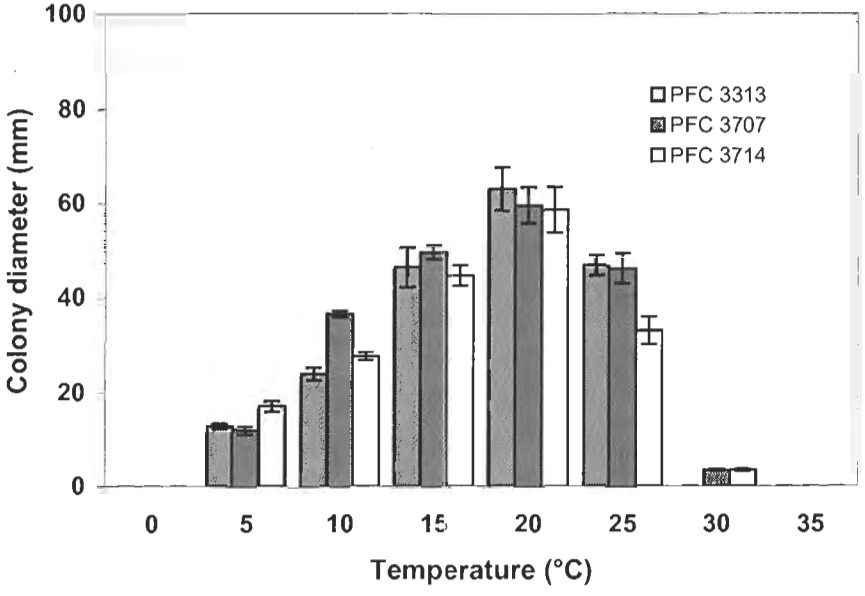


**Figure 2.1.** Effect of temperature on colony growth after 14 days of 3 *Phoma argillacea* isolates. Vertical bars represent standard error of the mean (n = 5).

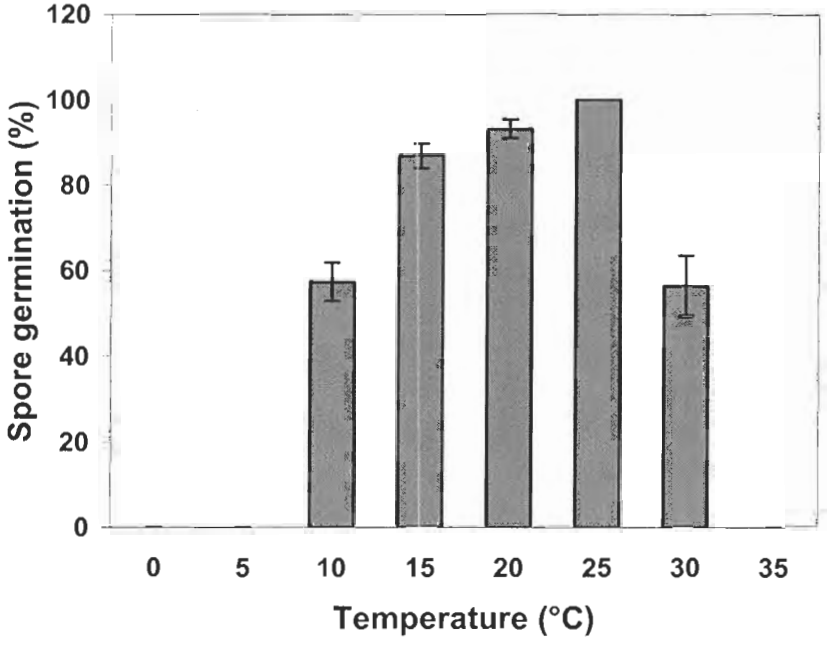


**Figure 2.2.** Effect of temperature on spore germination of *Phoma argillacea* (PFC 3313) after 24 hr. Vertical bars represent standard error of the mean (n = 1500).

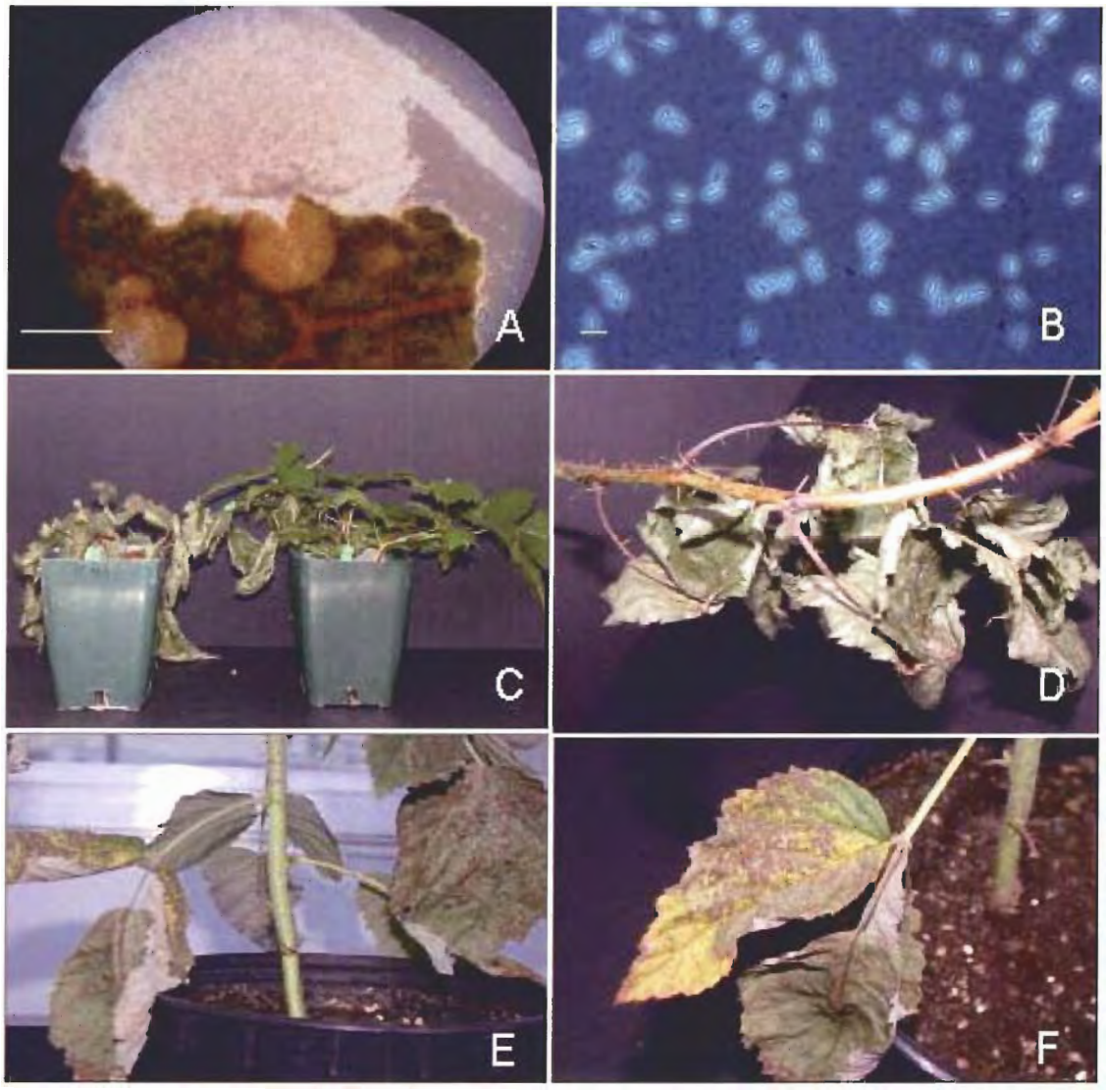


**Figure 2.3.** *Phoma argillacea* development on salmonberry following inoculation. A. Semi-immersed pycnidia on salmonberry leaves (scale bar = 100 μ); B. Pycnidiospores (scale bar = 10 μ); C. Disease symptoms on salmonberry leaves and branches following inoculations in the greenhouse. Note blighted and shriveled leaves. Right = control; Left = inoculated; D. Close- up of inoculated salmonberry leaves and branches following inoculations in the greenhouse; E, F. Disease symptoms on cultivated *Rubus idaeus* (red raspberry) following inoculations in the greenhouse.

**Table 2.3.** Effect of three *Phoma argillacea* isolates on disease severity (%) on salmonberry plants.

| | Disease severity* | | |
|---|---|---|---|
| Isolate | 7 days | 14 days | 21 days |
| PFC 3313 | 100 ± 0.0 a | 100.0 ± 0.0 a | 100.0 ± 0.00 a |
| PFC 3707 | 89.1 ± 7.1 a | 96.6 ± 2.6 a | 100.0 ± 3.11 a |
| PFC 3714 | 78.9 ± 11.1 a | 86.0 ± 7.0 a | 97.3 ± 2.0 a |
| Control (PDB) | 0.0 ± 0.0 b | 0.0 ± 0.0 b | 0.0 ± 0.0 b |

*A one-way analysis of variance comparing treatment means was performed. Within a column, means ± standard error of the mean followed by the same letter are not significantly different according to the Tukey-Kramer HSD test at $P = 0.05$.

**Table 2.4.** Comparison of mycelia and spore inoculum of three *Phoma* isolates on disease severity (%) on salmonberry plants.

| Inoculum | Disease severity* | | |
|---|---|---|---|
| | PFC 3313 | PFC 3707 | PFC 3714 |
| Mycelia | 97.2 ± 0.0 a | 96.6 ± 2.6 a | 78.9 ± 11.14 a |
| Spores | 79.2 ± 8.1 b | 89.2 ± 6.1 a | 21.0 ± 19.78 b |

*A one-way analysis of variance comparing treatment means was performed. Within a column, means ± standard error of the mean followed by the same letter are not significantly different according to the Tukey-Kramer HSD test at $P = 0.05$.

Previous research studies have revealed that using *S. rubi* (Wall and Shamoun 1990), *F. avenaceum, Colletotrichum dematium* (Pers.) Grove and *Phomopsis* sp. (Oleskevich et al. 1998) as potential biological control agents for *Rubus* species resulted in minimal control of salmonberry, with re-growth occurring after 3 weeks. In the present study, regeneration of salmonberry occurred 3 weeks after inoculation thus it may be necessary to make a second application of *P. argillacea* inoculum shortly after the appearance of new growth on the treated plants to further suppress salmonberry growth over one growing season. Split applications of a low dosage of herbicide followed by a biological control agent has been shown to enhance the efficacy of biological control agents by increasing disease symptoms and reducing plant dry weight (Bailey et al. 2000) and should be considered when applying *P. argillacea* under field conditions. Another alternative would be tank-mixing the biological control agent with a low rate of herbicide such as glyphosate, which was found to cause high level of control of fireweed with *C. dematium* under field conditions (Léger et al. 2001).

### 2.3.4. Effect of different leaf wetness periods on infection of salmonberry by *P. argillacea*

Under ambient greenhouse conditions and a 16-hr photoperiod, disease severity tended to increase with increasing leaf wetness periods (Figure 2.4A), while dry matter accumulation of the living aerial parts of salmonberry plants decreased with increasing leaf wetness periods (Figure 2.4B). A minimum leaf wetness period of 18 hr was required to cause significant disease (>80%). Leaf wetness periods of less than 18 hr did not produce any significant disease and there was no disease in the absence of leaf wetness (0 hr) after inoculation (data not shown). The average dry matter accumulation

of control plants was 9.6 g, which was not significantly different from plants receiving 3-12 hr of leaf wetness. However, dry matter was reduced by 57%, 73%, 68% and 71% compared to control plants when inoculated plants received 18, 24, 36 and 48 hr of leaf wetness, respectively (Fig. 2.4B).

### 2.3.5. Effect of inoculum concentration on infection of salmonberry by *P. argillacea*

A minimum inoculum concentration of 0.5% (v/v) ($3.5 \times 10^2$ CFU/mL) was required for mycelial suspension of *P. argillacea* to cause significant disease (>70% disease severity) (Figure 2.5A) on salmonberry plants provided with 48-hr leaf wetness in the greenhouse. However, plant growth was not significantly affected when less than 10% (v/v) ($2.6 \times 10^4$ CFU/mL) mycelia was applied (Figure 2.5B). This suggests that, for effective salmonberry control, a minimum mycelial inoculum of 10% (v/v) is required. Despite effective growth reduction, resprouting was observed on all treatments arising mostly from underground rhizomes.

### 2.3.6. Effect of filtrate on infection of salmonberry by *P. argillacea*

*Didymella applanata*, the teleomorph of *P. argillacea*, has been reported to produce an exocellular phytotoxic glycopeptide (Broekhoven et al. 1975; Lousberg et al. 1973) that induces chestnut brown lesions, ultimately killing cells. Treatment of salmonberry plants with *P. argillacea* filtrate did not cause foliar or stem necrosis, with no apparent phytotoxic symptoms compared to salmonberry plants inoculated with *P. argillacea* mycelia.

### 2.3.7. Effect of *P. argillacea* on non-target hosts

*Didymella applanata* is an ascomycetous fungus that produces bitunicate asci and belongs to the order Dothideales. It has been known for approximately 80 years that *D.*

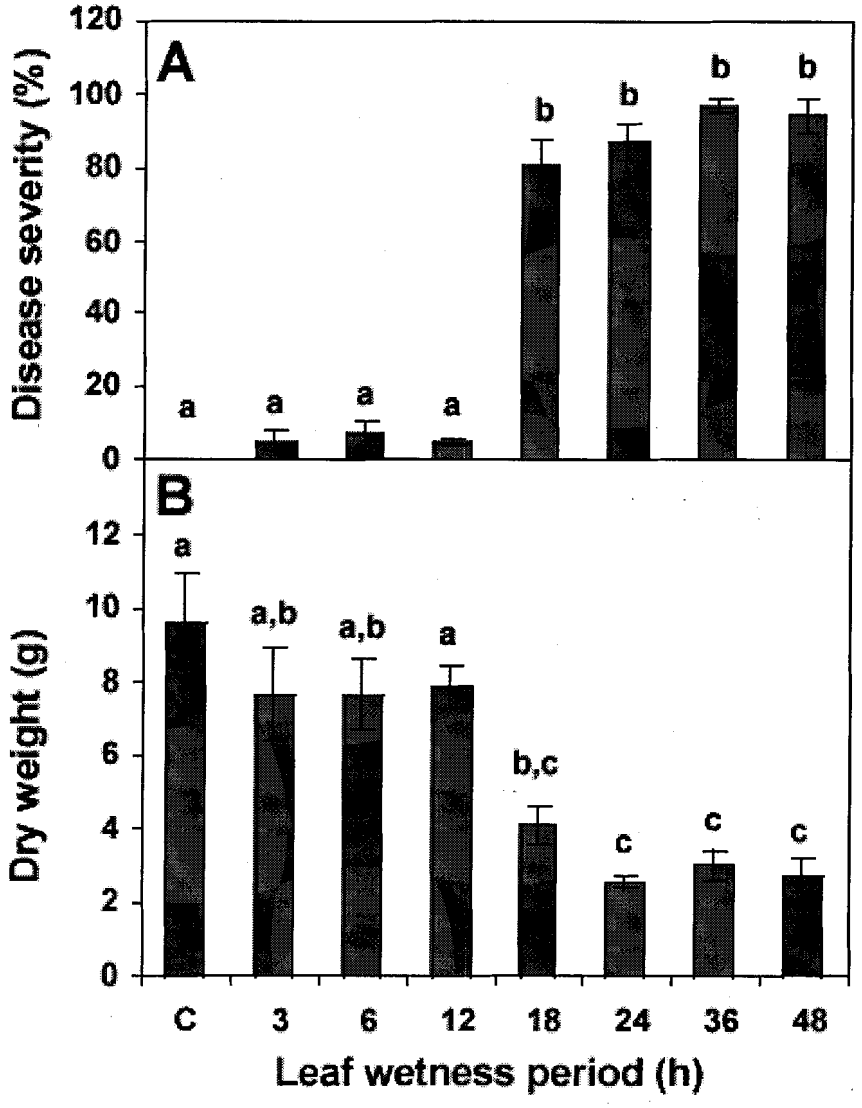


**Figure 2.4.** A. Effect of leaf wetness period on disease severity caused by *Phoma argillacea* (PFC 3313) on *Rubus spectabilis* after 7 days post-treatment; B. Effect of leaf wetness period on dry matter accumulation (g) by the living aerial parts of *Rubus spectabilis* plants 21 days after inoculation with *Phoma argillacea* (PFC 3313). The data points represent the average of five replications and the bars represent the standard error of the means. Bars identified by the same letter have means not significantly different according to the Tukey-Kramer HSD test at $P = 0.05$. C=control.

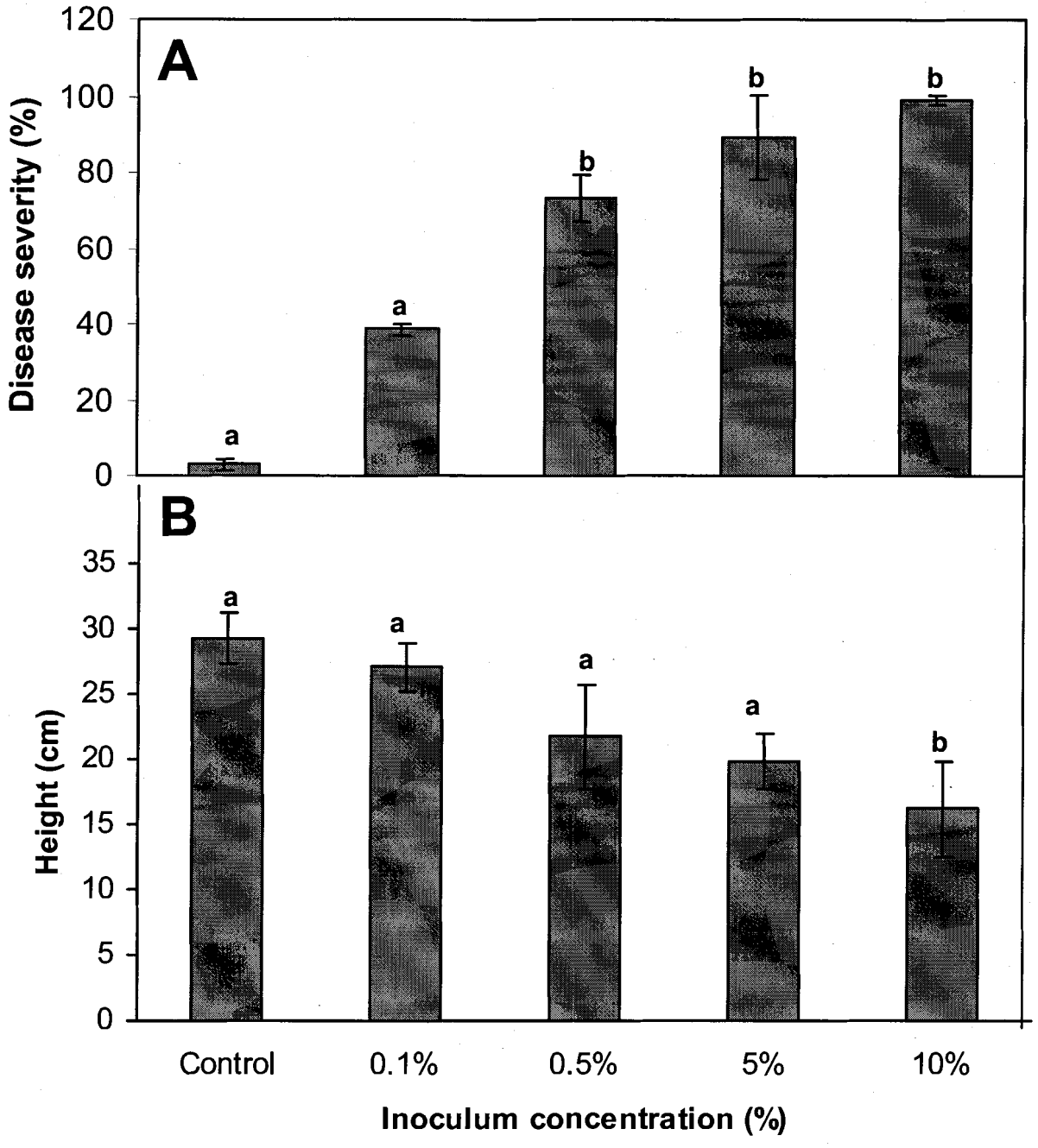


**Figure 2.5.** A. Effect of inoculum concentration (% v/v) on disease severity caused by *P. argillacea* (PFC 3313) mycelia after 21 days; B. Effect of inoculum concentration on plant height *R. spectabilis* after 21 days. The data points represent the average of 5 replications and the bars represent standard error of the means. Bars identified by the same letter have means not significantly different according to the Tukey-Kramer HSD test at $P = 0.05$.

*applanata* has a *Phoma* sp. anamorph (Koch 1931). The anamorphic state of *D. applanata* was recently named as *Phoma argillacea* (de Gruyter et al. 2002) and has been linked to its anamorph based on ITS sequence (Lindqvist-Kreuze et al. 2003; Reddy et al. 1998). Since my isolate was found to be 99.4% homologous to the submitted ITS sequence of *D. applanata,* the causal agent of spur blight on commercially important red raspberry, a greenhouse pathogenicity test was conducted using *P. argillacea* isolate PFC 3313. The results showed that *P. argillacea* caused limited leaf spots on red raspberries, developing as early as 4 days, but with no apparent damage to raspberry canes (Figures 2.3E, F). After 7 days, significant plant recovery was observed based on height recovery and development of new leaves that were disease-free (data not shown).

Since *P. argillacea* is being considered as a potential biological control agent for salmonberry in forest regeneration sites, conifer seedling tolerance to *P. argillacea* was tested. All three economically important conifers tested exhibited good seedling tolerance, showing less than 5% injury on needles under greenhouse conditions (Table 2.5). Red cedar was found to be the most tolerant, followed by western hemlock and Douglas-fir, respectively (Table 2.5).

Several fungi have been previously found to colonize salmonberry in its native location (Shamoun and Sieber 2000), but this is the first report of *P. argillacea* as a pathogen of salmonberry in British Columbia and Northwest USA (Conners 1967; Farr et al. 1989; Ginns 1986). However, its teleomorph *D. applanata* has been reported on two varieties of *R. idaeus* L. (red raspberry) and *R. loganobaccus* L.H. Bail. (loganberry) in B.C. (Fernando et al. 1999) and its occurrence has been reported in 10 Canadian provinces, except for Newfoundland and Prince Edward Island (Ginns 1986). *Didymella*

**Table 2.5.** Effect of *P. argillacea* (PFC 3313) on disease severity on Douglas-fir, red cedar and western hemlock seedlings after 35 days.

| Seedling type | % Disease severity* |
|---|---|
| Douglas-fir | 2.5 ± 0.60 a |
| Western hemlock | 2.2 ± 0.81 a |
| Red cedar | 0.2 ± 0.13 b |

*A one-way analysis of variance comparing treatment means was performed. Within a column, means ± standard error of the mean followed by the same letter are not significantly different according to the Tukey-Kramer HSD test at $P = 0.05$. n=15. Disease severity on conifer seedlings is defined as the percentage of infected needles/seedling.

*applanata* is distributed worldwide and it affects red raspberries throughout Europe and North America, but is rarely found on black raspberries or blackberries (Punithalingam 1982). It has also been isolated as an endophyte of apparently healthy-looking *R. parviflorus* Nutt. (thimbleberry) (Shamoun and Sieber 2000).

There are approximately 2000 putative *Phoma* species (Sutton 1980), but many of these are synonyms or are improperly placed within this genus. Currently, over 200 *Phoma* species are recognized and their distribution is worldwide. In North America, several *Phoma* species are known to be saprophytes or weak pathogens of numerous plants in agriculture and forestry (Morgan-Jones and Burch 1988; White and Morgan-Jones 1983; 1984; 1987). Several *Phoma* species have also been previously assessed as potential weed biological control agents, including *P. herbarum* Westend. and *P. exigua* (Desm.) to control dandelion (Neumann and Boland 2002; Stewart-Wade and Boland 2004) and salal (Zhao and Shamoun 2005), a *Phoma* sp. to control Canada thistle (Bailey et al. 2000), *P. proboscis* Heiny to control field bindweed (Heiny and Templeton 1991) and *P. clematidina* (Thüm.) Boerema to control old man's beard (*Clematis* sp.) (Gourlay et al. 2000). In the present study, *P. argillacea* was originally isolated from diseased salmonberry leaves and found to be pathogenic on detached leaves *in vitro* as well as on intact salmonberry plants grown in the greenhouse. Leaf and stem damage due to *P. argillacea* in greenhouse trials was >75% under environmental conditions that were conducive for mycelial growth and spore germination.

In addition to temperature and humidity, an extended period of foliar wetness was required for *P. argillacea* to infect the aerial parts of the target weed. The requirement for long leaf wetness periods has also been reported for other *Phoma* species, such as *P.*

*proboscis* on field bindweed, which required 12 hr or more of dew for high levels of disease to occur in the greenhouse (Heiny and Templeton 1991). The requirement of sufficient dew period is common to a number of potential mycoherbicides but might be overcome by formulation techniques that will either bypass or decrease the dew period requirement (Heiny and Templeton 1991). Several adjuvants have been used to enhance bioherbicidal efficacy of the biological control agents under field conditions (Neumann and Boland 1999; Rosskopf et al. 2000; Shabana et al. 1997) and may provide sufficient nutrition and protection to the pathogen.

Another important requirement for establishment of disease is inoculum concentration. Increasing the inoculum concentration often confers greater initial disease severity as reported on other plant-pathogen systems, such as the dandelion-*Phoma* system (Neumann and Boland 2002; Stewart-Wade and Boland 2004). This study showed that various dilutions of *P. argillacea* mycelia, when provided with sufficient leaf wetness period, caused high disease severity (>70%) and can be effectively used at low concentrations (0.5% v/v or $3.5 \times 10^2$ CFU/mL).

The principal objective of vegetation management is to suppress the target weed, reduce its competition with planted conifers for natural resources (i.e., moisture, nutrients, light) and allow release of conifer seedlings to a free growing state. This must be achieved within 1-3 yr after planting young conifer seedlings. The ability of *P. argillacea* to significantly reduce the dry weight of the aerial parts through defoliation and stem injury is encouraging. Prior to releasing *P. argillacea* as a biological control agent in reforestation sites, a detailed host range study must be conducted on non-target hosts, specifically conifer species, as well as other economically important crops. The

preliminary host range study has shown that several commercially valuable conifer seedlings, such as Douglas-fir, western red cedar and western hemlock, did not develop any significant disease symptoms when inoculated with *P. argillacea* mycelia applied as an aerial spray in the greenhouse. In addition, cultivated red raspberry was able to recover from initial infection and showed significant growth and emergence of new, disease-free shoots. These results are promising especially since the teleomorphic state of the fungus is known to cause spur blight on cultivated red raspberry. These findings warrant further investigation into formulation of *P. argillacea* to bypass the reliance on dew period and development of delivery technologies for management of salmonberry in young conifer regenerating sites.

# CHAPTER 3. GENETIC DIVERSITY OF *RUBUS SPECTABILIS* IN COASTAL BRITISH COLUMBIA

## 3.1. Introduction

### 3.1.1. Life history and vegetative growth

*Rubus spectabilis* Pursh. (salmonberry) is a densely growing perennial deciduous shrub native to North America and is a component of old growth understory in west coast forest stands (Klinka et al. 1996). It is a member of the Rosaceae family (genus *Rubus* subgenus *Idaeobatus*). Its range of distribution is west of the Coast Mountains, British Columbia from low subarctic/high temperate regions in the Aleutian Islands and southern Alaska, south to northwestern California (Oleskevich et al. 1995) and is a component of old growth understory in west coast forest stands (Klinka et al. 1996). Salmonberry colonization of new sites is primarily by seed dispersal and maintenance of a dense canopy can be attributed to its vegetative growth via extensive rhizome system. Establishment of salmonberry can substantially inhibit regeneration of trees and taller shrubs in clearcut or in regenerating sites (Tappeiner et al. 1993).

Salmonberry stems are connected via an underground rhizome system that creates an extensive clonal population structure. Clonal fragments include all stems (ramets) connected by rhizomes (Cook 1983). Salmonberry clone development is an active process involving growth of an underground rhizome into an area and subsequent ramet production from the rhizome bud bank (Tappeiner et al. 1991; Zasada et al.1992; 1994) which produces about 1-2 new rhizomes/yr extending 0.1-0.8 m/yr (Tappeiner et al. 1993). Ramets consist of a taproot and 1-5 aerial stems up to 4 m tall (Tappeiner et al. 1991), while rhizomes consist of well-defined nodes and internodes, with each node

having a preformed root primordium with density varying from 0.5 to 2 nodes per centimetre (Zasada et al. 1992). As individual clones (meaning those ramets that are still physically connected), salmonberry forms a loose network of rhizomes and a sparse canopy of aerial stems (Tappeiner et al. 1991) and covers areas of up to 35 $m^2$ (Tappeiner et al. 1991). In an excavation study, salmonberry populations were found to be composed of a coalescence of clones with intermixing of rhizomes below ground and aerial stems above, about two to six clones were found within 4-$m^2$ plots (Tappeiner et al 1991). Individual salmonberry clones were found to be fairly small compared to other reported shrub species. The maximum area for a single clone in Tappeiner et al.'s (1991) study was 50 $m^2$, and this area was occupied by other salmonberry clones as well. In comparison, maximum areas reported for other shrub species are 5 $m^2$ for beaked hazel (Tappeiner 1971), 10-20 $m^2$ for prickly rose (*Rosa acicularis* Lindl.) (Calmes and Zasada 1982), 150 $m^2$ for prickly ash (Reinartz and Popp 1987), 250 $m^2$ for sandbar willow (*Salix interior* Rowles) (Krasney et al. 1988) and 800 $m^2$ for smooth sumac (*Rhus glabra* L.) (Gilbert 1966). Clonal expansion, often measured on the basis of annual rhizome extension, is related to overstory conditions and was greatest in clearcut areas (Tappeiner et al. 2001) and varied among stand types (Tappeiner et al. 1991). Salmonberry clones in alder stands were larger (18 m total rhizome), as well as producing more ramets and more aerial stems, than those in conifer stands and on riparian sites (5-6 m rhizome) (Tappeiner et al. 1991).

### 3.1.2. Plant population genetics

Population genetics is the application of Mendel's laws and other genetic principles to entire populations of organisms (Hartl and Clark 1997). Natural populations

contain genetic variation in the form of multiple alleles of many genes and, for any specified allele, the allele frequency is the proportion of all alleles of the gene that are of the specified type (Hartl and Clark 1997). In the last two decades, there has been considerable research on population genetics of plants primarily due to the increased availability of molecular techniques suitable for plant genetics.

Genes in natural populations are either monomorphic or polymorphic. A polymorphic gene is (by convention) one for which the most common allele has a frequency of less than 0.95 (Hartl and Clark 1997). Genetic polymorphism, whether studied through allozymes or nucleotide sequences, provides a set of built-in markers for the genetic study of organisms in their native habitat (Hartl and Clark 1997). Mating system and mode of reproduction, as well as population size, can greatly affect the diversity and population structure of a species. Evolutionary forces that affect population genetic diversity and structure include: genetic drift, mutation, gene flow (referred to as migration among populations and dispersal within populations), and natural selection (McDonald and Linde 2002). These forces act individually and in concert to determine the genetic makeup and evolutionary potential of populations and therefore species. Between populations, the distribution of alleles are often assumed to be shared among populations because of migration, in which case allele frequencies can be used to estimate rates of migration. Within populations, alleles are shared through common ancestry (Hartl and Clark 1997). Gene diversity refers to the diversity at individual loci and is determined by the number and frequency of alleles. Genotype diversity refers to the frequency of unique genotypes or genetically distinct individuals. It is particularly relevant for clonal reproduction since there will be fewer genotypes than in a sexual

population, even though both the asexual and sexual populations could have the same number of genes and allele frequencies. Diversity can also be used to infer the age of the plant population and whether it was introduced or native.

Sexual reproduction through production of seeds is an important factor that generates genetic diversity within a plant species. It allows the flow of genes within and among populations. Sexually reproducing species often consist of populations comprised of multiple genets, while some plants that exhibit low or no sexual reproduction have populations that are comprised of only one or few genets. A genet is defined as the genetic individual that develops from the zygote and that produces ramets vegetatively, as well as stolons, rhizomes and bulbs.

In plants that exhibit rare recruitment of sexual offspring into established populations, significant genetic diversity is still maintained in these populations (Ellstrand and Roose 1987). This suggests that as long as a species can reproduce sexually even minimally, diversification of the genotypes within a species will be ensured. In extreme cases where plants produce sterile seeds or reproduce strictly via apomixis, the genetic variation is likely to have come exclusively from either mutations or multiple origins of the clones detected (Ellstrand and Roose 1987). Given the limited diversity obtained through somatic mutations, a reduction in seed and pollen dispersal could be deleterious if it results in a higher incidence of biparental inbreeding and inbreeding depression (Epperson 2000).

### 3.1.3. Population genetics of clonal plants

Since the main mode of growth and expansion in *R. spectabilis* is vegetative, it is expected that *R. spectabilis* will exhibit a predominantly clonal structure. Clonal

reproduction is a common phenomenon in plants. It can be achieved by vegetative spread, production of asexual propagules such as bulbils and joints, setting asexually produced seeds (agamospermy), or by permanent translocation heterozygosity (Ellstrand and Roose 1987). During clonal reproduction, new, morphologically distinct individuals have the original parental genotype and arise from somatic or germline tissues but have not undergone meiotic division (Orive 1993). Many clonal organisms are made up of physiologically integrated units, the ramets, which are originally genetically identical to each other and to their parents, and make up a single genet having been derived from a single zygotic ancestor (Orive 1993; Corradini et al. 2002; McLellan et al. 1997).

Many plant species can produce offspring that are genetically identical to each other and to the maternal plant, and are products of asexual reproduction or selfing. Mutations may create new, more virulent alleles, which, in the presence of selection, may increase in frequency in the population, disrupting the present equilibrium of resistance and virulence (McDonald and Linde 2002). A small amount of gene flow and/or mutation must occur to add new genotypes to a population from time to time in order to maintain genetic variation in clonal plants (Ellstrand and Roose 1987). However, mutations can also increase the number of deleterious alleles in the population over time, reducing its overall fitness. Without recombination to remove deleterious alleles and shuffle genotypes, it is thought that strictly clonal populations will eventually go extinct, due to the lethal combination of deleterious alleles and drift (Kohn 1995). Lynch and Gabriel (1990) predicted that the maximum persistence of clonal populations is $10^4$ to $10^5$ generations. This persistence is dependent on the success of a particular genet's ramets (Orive 1993). This means that studying the spatial connection, as in ramets and

rhizomatous stands, could be helpful in identifying the clone's limits and ancestry. Further, it is also crucial that somatic mutations occur to maintain genetic diversity (Orive 1993), allowing diversification of individuals that were once of the same genotype but over time has generated enough mutations to become an individual with a different genotype. Clonal populations often have small effective population sizes, increasing the relative impacts of genetic drift and population bottlenecks due to extinction. Assuming finite populations, genetic drift will eventually result in allelic loss or fixation, decreasing heterozygosity and reducing evolutionary potential (McDonald and Linde 2002).

Traditionally, strictly clonal plants were thought to have low genetic diversity, but more recently, several clonal plants have shown a high number of genotypes with moderate levels of diversity and an even distribution of genotypes (Ellstrand and Roose 1987). Comparative studies of related species with varying modes of reproduction revealed no significant difference in mean genetic variation (Campbell et al. 1999). The clonal plant *Calamagrostis porteri*, which exhibits reduced sexual reproduction due to rare flowering, low seed initiation and high embryo abortion (Esselman et al. 1999), was found to have significant levels of diversity as detected by nuclear DNA markers. This diversity is thought to originate from founder seeds and continual seedling recruitment (Eriksson 1993). As well, competition among clones, diversifying selection, and frequency-dependent selection can all result in high local heterogeneity, which in turn leads to high genotypic diversity across the populations (Ellstrand and Roose 1987). However, it can also be argued that generation times for clonal organisms can be quite large and a population of clonal plants may exhibit high genetic diversity because the plants may be very old and some of the diversity detected represents segments of DNA

within noncoding regions (Esselman et al. 1999). Many clonal plants could be characterized as weedy; therefore, if colonization occurs by multiple genotypes, the nature of weedy populations may preclude significant loss of genetic variation while these populations are extant (Ellstrand and Roose 1987). Diversity with clonal populations can be affected by the type of growth, such as phalanx or guerilla; however, Tappeiner et al. (2001) showed that clonal expansion of *R. spectabilis* is more dependent on overstory cover and competition among clones than a particular clonal growth strategy.

Population genetic analyses often partition up the genetic and genotypic diversity within and among populations. The level of clonality and distribution of clones can also be used to assess population structure. Clonal population structure can greatly affect the dynamics of plant populations and can influence a species' ability to compete with other plants and fight disease (Godt and Hamrick 1999). Understanding the population structure of *R. spectabilis* can help predict the possible effects of releasing a biological control agent on natural populations.

### 3.1.4. Genetic markers

Several molecular markers are available that are able to address plant genetic diversity and population structure. Genetic markers are used to quantify the genetic variation present in a population and the type of marker chosen can greatly affect the type of information obtained and the analyses that can be performed. Genetic markers can be separated into two general groups: those that are under selection, such as pathogenicity and resistance phenotypes, and those that are considered selectively neutral. The former are highly relevant to breeding systems and are more likely to provide biased estimates of

the potential for genetic change due to selection, and the results can be confused by linkage disequilibrium between loci and hitchhiking alleles (McDonald and McDermott 1993). Selectively neutral markers are typically chosen for population genetic studies as they are the most suitable for answering questions about overall diversity, population structure, and gene flow. When choosing a marker system, markers should be selectively neutral, highly informative, reproducible, technically simple, and cost efficient.

The following is an overview of the common genetic markers used in genetic diversity studies discussing some its advantages and disadvantages, describing the characters of some genetic markers that can be used and the method that was chosen for this study. In choosing a marker system for *R. spectabilis*, it was important to consider what, if any, previous research had already been done, the time and resources available, and the goals of this study.

Ideally, codominant markers in which heterozygous individuals can be distinguished from homozygous individuals should be used, such as allozyme variation in isozymes. Allozyme markers produce a positive correlation between the amount of polymorphism and the degree of heterozygosity. However, because they are functional enzymes, some loci may be under selection and the amount of variation observed is limited, resulting in an underestimation of genetic diversity in plant populations as evidenced by comparative studies of allozymes and DNA markers (Esselman et al. 1999). It may underestimate the amount of polymorphism because conventional electrophoresis fails to detect many amino acid replacements (Hartl and Clark 1997). On the other hand, it is also believed that allozymes might overestimate the amount of polymorphism because the enzymes typically surveyed are those found in relatively high concentration

in tissues (Group I enzymes) and often lack the high substrate specificity of enzymes implicated in central metabolic processes (Group II enzymes) (Hartl and Clark 1997).

The greatest limitation of protein electrophoresis is the inability to detect variation in nucleotide sequences that do not alter the amino acid sequence. Silent polymorphisms and polymorphisms in noncoding regions fall into this category. A polymorphism is silent if it is present in the coding region but does not alter the amino acid sequence; many nucleotide differences in the third codon position are of this type (Hartl and Clark 1997). A polymorphism is noncoding if it affects nucleotides in noncoding regions such as the upstream region, the downstream region, or introns (Hartl and Clark 1997). Examples of these include organellar DNA markers (i.e., chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA)) and nuclear DNA markers (i.e., randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP)).

Restriction fragment length polymorphisms (RFLPs) are also codominant markers. While they are generally more variable than allozymes as they survey both coding and noncoding regions of DNA, they are more technically challenging and expensive to perform (McDonald and McDermott 1993). As well, RFLPs are used mostly in phylogenetic and mapping studies, with known parent and offspring, and are most informative when controlled crosses can be done and parents are known, which is not readily feasible for *R. spectabilis* since it is mainly clonal in growth.

Nuclear DNA markers, coupled with PCR, are more more useful in genetic diversity studies because they detect a greater number of characters, hence more genetic diversity will be encountered (Ellstrand and Roose 1987). The availability of a variety of DNA markers during the last decade has enabled researchers to use them in population

genetic studies in natural populations such as forest plants (Deshpande et al. 2001), rare and endangered plant species such as *Calamagrostis porter* ssp. *insperata* (Esselman et al. 1999), as well as in introduced plants (Pappert et al. 2000) and cultivated species such as American ginseng (Schluter and Punja 2002). Microsatellites or simple sequence repeats (SSRs) are another codominant marker which are becoming increasingly popular in molecular studies. These markers are often highly polymorphic and informative since SSRs are usually found in highly variable regions of the genome. However, the primers are mainly species-specific, so unless they have been previously developed, SSR markers are often too expensive since primers must be developed and genomic libraries sequenced (Mueller and Wolfenbarger 1999). In addition, Hardy (2003) showed that in some cases, dominant markers can be equivalent to or better than SSRs at estimating spatial genetic structure within populations.

Conversely, dominant markers can also be used to study genetic diversity; however, with dominant markers, heterozygotes cannot be distinguished from dominant homozygotes, which is often considered a drawback and considered less suitable for linkage analysis than codominant markers (Hansen et al. 1998). Dominant markers segregate in a Mendelian fashion (Campbell et al. 1999). Examples of dominant markers include amplified fragment length polymorphism (AFLP), intersimple sequence repeats (ISSR) and randomly amplified polymorphic DNA (RAPD).

Amplified fragment length polymorphism (AFLP) uses dominant markers to genotype individuals (Vos et al. 1995). In this case, restriction enzymes are used to digest the DNA and long primers are used to amplify fragments. As such, AFLPs require no prior sequence knowledge and can generate a large number of loci from each primer

set, thereby requiring fewer primers (Mueller and Wolfenbarger 1999). However, AFLPs are considered more technically challenging and expensive.

The marker that I used to study salmonberry genetic diversity was RAPD. Random amplified polymorphic DNA (RAPD) is another dominant marker, which uses short (ten base pair) oligonucleotide primers of arbitrary sequence and low stringency PCR to amplify arbitrary regions throughout the genome, requiring no prior sequence knowledge (Williams et al. 1990). Because of the arbitrary sequence of the primers, RAPDs are usually highly variable, very quick and easy to develop and inexpensive to use. When employed with methodological care and interpreted conservatively, RAPD markers are reliable sources of genetic data in studies of closely related individuals (Campbell et al. 1999). In many plant groups, RAPDs have recorded similar levels of genetic diversity as RFLP and allozymes (Campbell et al. 1999). Specifically, RAPD markers have been used to measure genetic diversity within and between populations in a number of plant species, including *Rubus* (Trople and Moore 1999). They have also been used to compare genetic diversity between agamospermous and sexual species of *Amelanchier* (Campbell et al. 1999) and among natural and cultivated field populations and seedlots of American ginseng (Schluter and Punja 2002). However, RAPD markers also have many drawbacks, which include difficulties in reproducibility and relatively few loci per primer (Bornet and Branchard 2001).

### 3.1.5. Objectives

The aim of this study was to investigate the population genetics of salmonberry to determine the amount and distribution of genetic diversity in natural populations in British Columbia.

## 3.2. Materials and Methods

### 3.2.1. Site description

Salmonberry was collected from reforested sites in Jordan River (JR), Port Alice (PA), and Gold River (GR) on Vancouver Island, and Stafford Lake (SL) and Powell River (PR) on coastal British Columbia (Figure 3.1). Localities sampled and their geographical coordinates are summarized in Table 3.1. Samples collected from one site were considered to represent one population.

### 3.2.2. Sample collection

All five populations were sampled in exactly the same fashion. Briefly, 49 individuals separated by at least 15 m within a 90 m x 90 m plot were selected from each site. Young, healthy leaves were obtained from one individual, immediately stored in plastic vials containing silica gel, and stored at 4°C until DNA isolation was conducted.

### 3.2.3. DNA isolation

For DNA isolation, approximately 10 mg silica-dried leaf material was ground in 1.7 mL Eppendorf tubes with liquid nitrogen using micropestles. The isolation protocol for this study was a CTAB method for isolation of plant DNA (Doyle and Doyle 1987) with the following modifications: CTAB buffer (2% CTAB, 2% PVP, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris-HCl (pH 8.0), 0.5% β-mercaptoethanol added just before use) was pre-heated at 65°C and 600 μL was added to each sample; ground samples with hot CTAB were ground for another 30 seconds, incubated for 2.5 hours at 65°C, gently mixing every 15 minutes, and centrifuged for 1 minute at 14000 rpm (room temperature 22°C) (Eppendorf Centrifuge 5417R, Hamburg, Germany). During chloroform

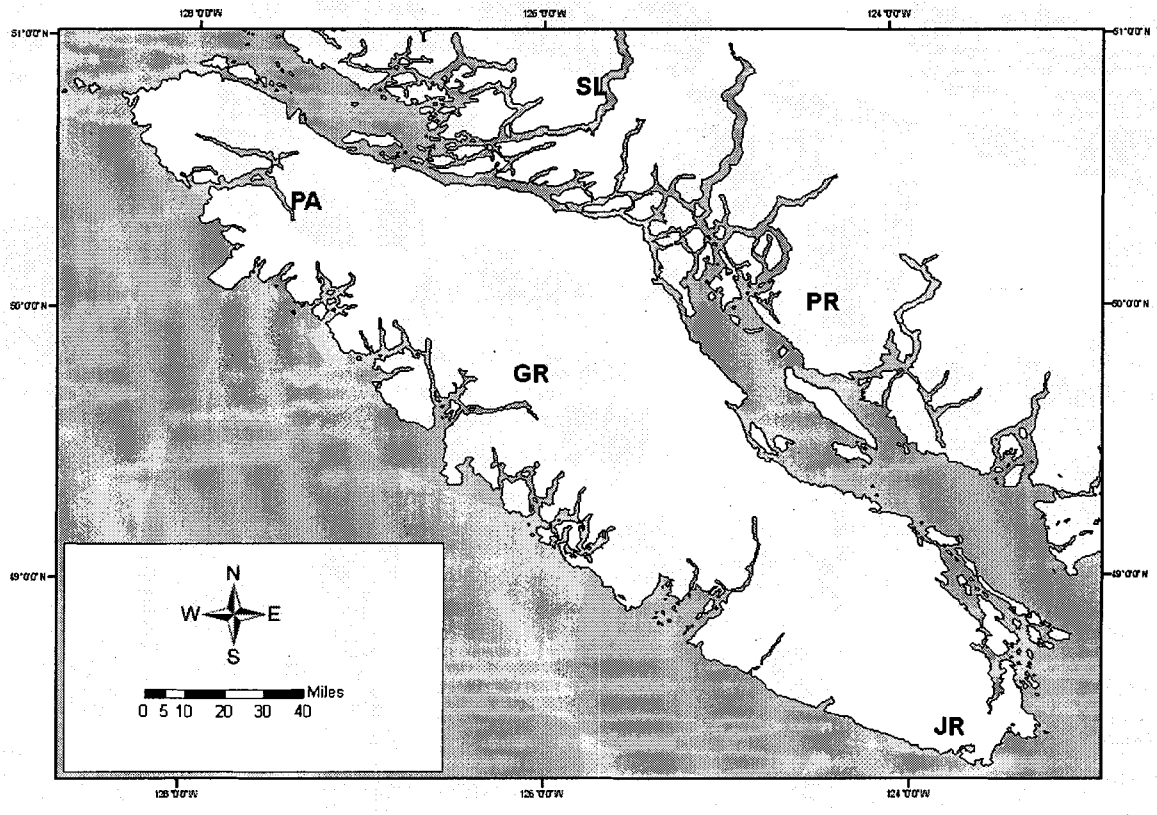


**Figure 3.1.** Map of Vancouver Island and mainland British Columbia, showing the locations of the five salmonberry populations used in this study: Jordan River (JR), Port Alice (PA), Stafford Lake (SL), Powell River (PR) and Gold River (GR). Map courtesy of Kangakola Omendja.

**Table 3.1.** Locations and descriptions of five collections sites where young salmonberry leaves were collected .

| Site | Date (mm/dd/yy) | Location | Latitude and longitude |
|---|---|---|---|
| 1 | 29/04/02 | Jordan River, BC | 49°15'42"N 124°13'58"W |
| 2 | 07/05/02 | Port Alice, BC | 50°16'13"N 127°24'01"W |
| 3 | 08/05/02 | Stafford Lake, BC | 50°49'18"N 125°25'17"W |
| 4 | 09/05/02 | Powell River, BC | 49°46'21"N 124°18'36"W |
| 5 | 10/05/02 | Gold River, BC | 49°50'55"N 126°21'36"W |

extraction, an equal volume (600 μL) of CHL:IAA extraction buffer (24:1 chloroform:isoamyl alcohol) was added by inverson for 10-12 minutes or by flicking until the two phases obtained a milky appearance;layers were separated by centrifuging tubes at room temperature for 5 minutes at 14,000 rpm, the aqueous phase (~400 μL) was transferred to new tubes and the extraction was repeated. The second aqueous phase (~300 μL) was transferred to a 1.5 mL Eppendorf tube and incubated at room temperature for 30 min with 10X Rnase A (1 mg/mL, Sigma, Oakville, ON, Canada); DNA was precipitated with 0.08X 7.5 M ammonium acetate, 0.54X ice-cold isopropanol, mixed by flicking with finger followed by a 10-minute incubation at room temperature and centrifugation at 14,000 rpm at room temperature to obtain a pellet. Following DNA precipitation with isopropanol, the supernatant was poured off and the pellet washed with 1 mL 70% ETOH by inverting the tube 2-3 times to wash the internal surface of the tube. DNA was pelleted at 14,000 rpm for 5 min at 4°C and the air-dried pellets were resuspended overnight at 4°C in 50μL 1X TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was isolated from two replicates of each salmonberry sample and then combined for a final volume of 100 μL TE. The concentration and quality of DNA was determined using 1.2% agarose gel electrophoresis in 1X TBE and by measuring the $A_{260}/A_{280}$ ratio with a spectrophotemeter (Pharmacia Biotech, Amersham, Piscataway, NJ, USA). DNA was diluted to 1:10 for the RAPD-PCR reactions.

### 3.2.4. PCR reactions

PCR reactions were carried out according to Graham and McNicol (1995) with modifications. Briefly, PCR reactions were carried out in 25-μL volumes containing per reaction, 1 μL genomic DNA (10X), 1.25 μL dNTP solution (2 mM) (Amersham

Biosciences), 0.5 μL primer (10 μM), 2.5 μL of 10 X PCR buffer (Biocan), 2.0 μL $MgCl_2$ (25 mM), and 0.2 μL Gold Taq (5 U/μL) (Biocan). PCR reactions were carried out in an Applied Biosystems GeneAmp PCR System 9700 under the following conditions: 45 cycles at 92°C for 1 min, 35°C for 3 min, and 72°C for 5 min. Random primers were obtained from Qiagen and from the NAPS Service at the University of British Columbia.

### 3.2.5. RAPD analysis

To find primers for salmonberry, the literature was surveyed for RAPD primers previously used on *Rubus* species (Alice and Campbell 1999; Campbell et al. 1999; Graham and McNicol 1995). Ten 10-mer RAPD primers were selected from the literature survey as well as an additional 20 10-mer RAPD primers from Operon (Kit B, Qiagen). A total of 30 RAPD primers were subsequently tested on salmonberry. Primers producing clear banding patterns and polymorphisms were selected for use. To ensure reproducibility, the procedure was independently repeated on a reduced sample set (5 samples) from five populations.

### 3.2.6. Assumptions and scoring of RAPD gels

When scoring RAPD fingerprints, it was assumed that comigrating bands on a gel were identical in sequence and that repeatable loci represented stable fragments. Given the predominantly vegetative growth of salmonberry, Hardy-Weinberg (H-W) equilibrium could not be assumed. As such, the Lynch and Milligan (1994) correction factor for diploid species with dominant markers was not used to calculate allele frequencies or heterozygosity. Instead, the dominant allele frequency was calculated as $p = x/N$ where x is the number of individuals with the marker and N is the total number

of individuals. This inflates the frequency of the dominant allele by masking the contribution of heterozygotes, leading to an underestimate of gene diversity (Lynch and Milligan 1994). When scoring the RAPD gels for presence or absence of bands, individuals showing no band at a locus were considered absent (null), and those with a band (irrespective of intensity) were considered present. It was therefore assumed that if there was no band present, the individual was homozygous recessive and if there was a band, the individual was assumed to have at least one copy of the dominant allele. For a diploid, the presence of a band could indicate a heterozygote (one null and one dominant allele) or homozygous dominant (two copies of the dominant allele).

### 3.2.7. Data analysis

RAPD gels were scored for presence or absence of bands. Based on frequency of the dominant (present) allele, loci with frequencies >0.95 and <0.05 were trimmed to reduce the statistical error and variance introduced by these loci (Hardy 2003). Individuals with missing data were not included in the analyses as they biased pairwise comparisons. Average gene diversities and heterozygosities were calculated across all loci from the square root of the frequency of the null genotype ($q^2 = N_{null}/N_{total}$), assuming a dominant, diploid system (Hendrick 2000). Fingerprints were compared within and among populations using Arlequin Version 2.000 (Schneider et al. 2000) to determine the number of pairwise mismatches between individuals. The observed mismatch distribution was also compared to expected distributions based on a step-wise expansion model using a parametric bootstrap approach in Arlequin.

Differentiation among salmonberry populations was determined by analysis of molecular variance (AMOVA) analysis (Excoffier et al. 1992) using Arlequin. A matrix

of population pairwise genetic distances ($F_{ST}$) and number of mismatched loci between populations was also obtained. The pairwise $F_{ST}$ values were used to create a dendogram using an unweighted pair group method with arithmetic mean (UPGMA) in NTSYSpc 2.1.

### 3.3. Results

Screening of RAPD primers resulted in the selection of four primers after the reproducibility test was performed. The four primers used to generate RAPD fingerprints resulted in a total of 40 scorable loci. Some individuals had missing data (i.e., failed to amplify for one or more primers). Removal of individuals with missing data from the analysis resulted in the reduction of total sample size from 245 to 117 individuals. The percentage of polymorphic loci combined over all populations was found to be 92.5% (Table 3.2). The average percentage of polymorphic loci within populations was 75.5%. The Gold River (GR) population had the lowest diversity (70.0% polymorphic loci) compared to Port Alice (PA) and Powell River (PR) where 80.0% of the loci were polymorphic (Table 3.2). However, dominant alleles with frequency of >0.95 and <0.5 were removed to reduce the statistical error and variance introduced by these loci (Hardy 2003). Once loci with >0.95 and <0.05 were removed, it resulted in 35 loci being used for population-level analyses (Table 3.3).

#### 3.3.1. Genetic diversity

Average genetic diversity across all populations was calculated using the adjusted 35 loci (Table 3.3). Due to the dominant nature of RAPD markers and because salmonberry is diploid, the frequency of the recessive locus can be estimated from the square root of the observed frequency of the null genotype in populations. In this case,

**Table 3.2.** Sample size, number of monomorphic loci, and % polymorphic loci in each population based on 40 loci, where the dominant allele refers to the presence of a band and the recessive allele refers to the absence of a band.

| Population | Sample size[1] | No. monomorphic loci | | % Polymorphic loci |
|---|---|---|---|---|
| | | No. dominant | No. recessive | |
| JR | 19 | 7 | 4 | 72.5 |
| PA | 33 | 6 | 2 | 80.0 |
| SL | 19 | 8 | 4 | 75.0 |
| PR | 18 | 4 | 4 | 80.0 |
| GR | 28 | 7 | 5 | 70.0 |
| Total | 117 | 3 | 0 | 92.5 |

[1]Individuals with missing data were excluded.

**Table 3.3.** Number of loci obtained from each 10-mer RAPD primer for all populations and number of loci used in among-population analysis, after pruning loci with very high or low frequencies[1].

| Primer | 5' to 3' | Total no. loci | No. loci used for all pops |
|---|---|---|---|
| OPB4 | GGACTGGAGT | 9 | 7 |
| OPB7 | GGTGACGCAG | 10 | 10 |
| UBC5 | TGCGCCCTTC | 11 | 10 |
| UBC7 | CCACCGCCAG | 10 | 8 |
| Total | | 40 | 35 |

[1]Loci with dominant frequencies >0.95 and <0.5 were not included in the analyses.

across all loci in all populations, the observed frequency of the recessive, null genotype ($q^2 = N_{null}/N_{total}$) was 0.56, resulting in an average gene frequency of the null allele ($q$) of 0.75. Subtracting $q$ from 1 gives 0.25, the average frequency of the dominant allele ($p$) and an average heterozygosity of 0.38, calculated as $H=2pq$, indicating that 38% of the individuals in the populations are heterozygous, with at least one copy of a null allele. No two individuals had identical fingerprints within or between populations and the percentages of polymorphic loci were high.

The number of pairwise differences between individuals within each population, ranged from 8.6 (GR) to 9.7 (JR) mismatches between individuals (Table 3.4). In some cases pairs differed by only a few loci, and could therefore be considered as belonging to the same clone. The $p$-values for the mismatch distributions from each population were not significant; therefore, the null hypothesis that there are fewer differences observed than expected could not be rejected. The result suggests that the observed number of differences between individuals within each population is less than expected from a randomly mating population. Therefore, the result provides support that some level of clonality is occurring within populations of salmonberry.

### 3.3.2. Differentiation among populations

To assess the level of differentiation between salmonberry populations, an AMOVA was performed. The AMOVA resulted in an $F_{ST}$ of 0.0454 where 95.46% of the variation was partitioned within the populations and 4.54 % of the variation was explained by differences among populations (Table 3.5). Using Wright's (1943) island model, gene flow can be estimated from $Nm = 1/4[(1/F_{ST}) - 1]$ (Slatkin 1987), suggesting that there are on average 5.3 migrants per generation between populations.

**Table 3.4.** Number of pairwise mismatches between individuals within each population, showing the observed variance, minimum and maximum number of mismatches between pairs, and the *p*-value obtained from comparing observed and expected mismatch distributions.

| Population | No. pairs[1] | Ave. no. mismatches | Minimum no. mismatches | Maximum no. mismatches | Observed variance | *P*-value[2] |
|---|---|---|---|---|---|---|
| JR | 171 | 9.6 | 7.1 | 11.2 | 9.3 | 0.69 |
| PA | 528 | 9.7 | 8.3 | 11.4 | 9.8 | 0.30 |
| SL | 171 | 9.6 | 7.7 | 11.7 | 9.7 | 0.49 |
| PR | 153 | 8.8 | 7.5 | 11.3 | 9.4 | 0.10 |
| GR | 378 | 8.6 | 7.5 | 10.4 | 8.7 | 0.73 |

[1]Number of pairs determined by n(n-1)/2, where n is the population size.

[2]*P*-value is for the null hypothesis where the expected mismatch distribution, calculated using a step-wise expansion model, is greater than or equal to the observed distribution based on a parametric bootstrap approach (Schneider et al. 2000). Significant *p*-values ($\leq 0.05$) would reject the null hypothesis and indicate that more differences were observed than expected from the simulated distribution.

**Table 3.5.** Analysis of molecular variance (AMOVA) for five salmonberry populations resulted in an $F_{ST}$ of 0.0454. All tests were highly significant ($p$<0.0001) based on 1023 permutations[1].

| Source | d.f. | Sum of squares | Variance components | % of variation |
|---|---|---|---|---|
| Among populations | 4 | 38.836 | 0.221 | 4.54 |
| Within populations | 111 | 519.044 | 4.634 | 95.46 |
| Total | 116 | 557.880 | 4.855 | |

[1]AMOVA was performed with all 5 salmonberry populations included.

The matrix of population pairwise $F_{ST}$ values in Table 3.6 suggests that GR and PA are the least differentiated populations ($F_{ST}$=0.02844). As well, Powell River and Stafford Lake populations are also very similar ($F_{ST}$=0.02937) compared to PA and JR, which had the highest $F_{ST}$ of 0.06450 (Table 3.6). UPGMA analysis with the population pairwise $F_{ST}$ values resulted in a dendogram showing the genetic distances (Figure 3.2). Populations SL and PR formed a cluster, PA and GR formed a second cluster, and JR was in the third cluster (Figure 3.2).

The populations show low differentiation based on the AMOVA; however, qualitative comparisons of the pairwise $F_{ST}$ values and the UPGMA dendogram suggest there may be some isolation by distance (Table 3.6, Figure 3.2). For example, PA is most genetically similar to GR, which is the closest population geographically, and most genetically differentiated from JR, which is the southernmost population. This is also evidenced with SL being clustered with its most similar population PR, and again most genetically differentiated from JR.

## 3.4. Discussion

### 3.4.1. Genetic diversity

In general, populations of clonal plants exhibit considerable levels of genetic diversity (Ellstrand and Roose 1987; Hamrick and Godt 1997). Extremely high genetic diversity was observed within and among all five salmonberry populations in this study as determined from the percentage of polymorphic loci. The average within population percent polymorphic loci was 75.5%, and the total percent polymorphic across all populations was 92.5%. As well, the average heterozygosity across all populations was 0.38. These results are similar to those found on studies with other rosaceous species

**Table 3.6.** Matrix of population pairwise genetic distances ($F_{ST}$) values for salmonberry populations corrected for sample size.

| Population | JR | PA | SL | PR | GR |
|---|---|---|---|---|---|
| JR | ----- | | | | |
| PA | 0.06450 | ----- | | | |
| SL | 0.06293 | 0.03484 | ----- | | |
| PR | 0.03191 | 0.05284 | 0.02937 | ----- | |
| GR | 0.04441 | 0.02844 | 0.05041 | 0.05806 | ----- |

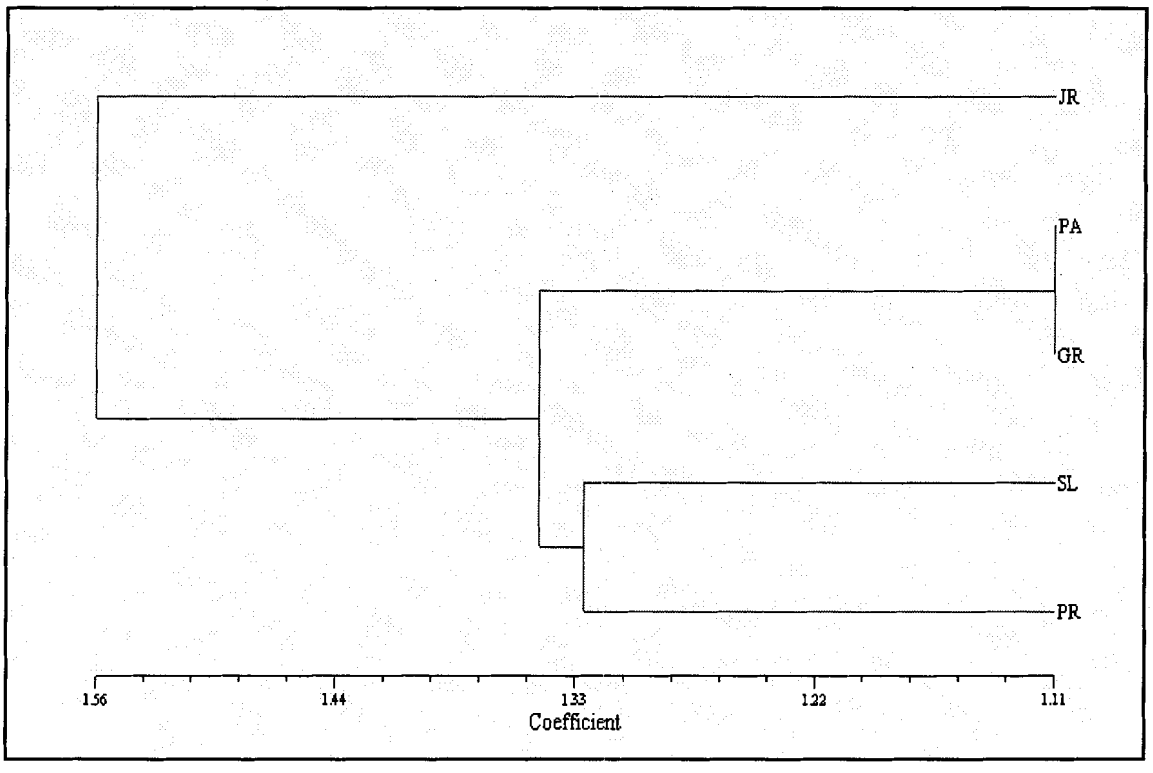


**Figure 3.2.** UPGMA dendogram of salmonberry populations based on pairwise $F_{ST}$ genetic distances. Letters represent geographical locations of the individual populations in British Columbia identified in Figure 3.1.

(Pluess and Stöcklin 2004). Using RAPD-PCR on *Geum reptans* L., Pluess and Stöcklin (2004) revealed an average 71.33% polymorphism. The mean molecular variation within populations (average heterozygosity of 0.38) was slightly, but not significantly, higher than the mean of 41 RAPD studies reviewed by Nybom and Bartish (2000) ($H_e = 0.21 \pm 0.12$). These results support the statement of Hamrick and Godt (1989) that, in general, clonal plants are genetically as diverse as nonclonal plants (Ellstrand and Roose 1987).

### 3.4.2. Differentiation among populations

Analysis of molecular variance (AMOVA) showed little population differentiation among the salmonberry populations used in this study. An overall $F_{ST}$ of 0.0454 was observed, which is comparable to the level of population differentiation observed for other long-lived woody perennial species (0.084; Hamrick et al. 1992). Low population differentiation in salmonberry could be explained by high gene flow resulting on a mixing of the gene pools among populations. Assuming the island model, an estimated 5.3 migrants would be necessary to maintain the level of differentiation observed. This level of migration could be easily achieved given salmonberry's mode of pollen and seed dispersal, especially among the populations used in this study, which were all from a relatively localized geographic region.

From population pairwise $F_{ST}$ values, GR and PA appear to be the most related population (smallest $F_{ST}$; 0.02844) and there is some evidence of isolation by distance from the dendogram (Figure 3.2). For example, PR and SL were geographically close to each other and had similar low $F_{ST}$. Populations PA and GR, located northeast of Vancouver Island, were clustered closer to PR and SL, which is on the mainland, compared to JR, which is the most distant population geographically. The closer

relationship between PR and SL and PA and GR could be explained by wind patterns, effectively shortening the distance between mainland and island populations, or by a founder effect. This north-south partitioning of populations based on observed genetic differentiation has been observed for many plant and animal species of the Pacific Northwest (Soltis et al. 1997).

High levels of gene flow could explain the low levels of population differentiation observed, although if this was the case, highly related individuals should have been observed among populations. Another explanation for low population differentiation could be sampling strategy. The sampling may not have been truly representative of clones in the natural populations and could directly impact the results, as was suggested by Nybom and Bartish (2000). Low differentiation could also be due to the RAPD markers, where perhaps different loci or a different marker system would have revealed more differentiation among populations. Another possible explanation is related to the geographic distribution of salmonberry, which is limited to the Pacific Coast from northern California to southern Alaska. The populations used for this study are from a relatively small area in the center of the species range, which could explain the high diversity observed within populations and the low differentiation between populations. If the populations were further apart, for example from the edges of the range, it is likely they would be more differentiated.

The fact that high gene flow occurs among *R. spectabilis* populations, as exhibited by the high diversity within population and low population differentiation, has implications for biological control. It would be preferable to have differentiated populations with very low gene flow as this would effectively restrict the biological

control agent to the target area (Wilkin et al. 2005). In addition, highly diverse populations may also pose a problem in finding one biological control agent, which is effective over one whole population. However, if the biological control agent selected for control of *R. spectabilis* was successful in controlling one highly diverse population of *R. spectabilis*, control of other highly diverse populations is very likely to occur. Preliminary greenhouse studies using *P. argillacea* and one representative clone of *R. spectabilis* from all five populations showed no significant difference in disease severity among populations (unpublished data). Obtaining more representative clones from each of the five populations and subsequently testing their relative susceptibility to *P. argillacea* under greenhouse conditions would provide further information with respect to the correlation between genetic diversity and susceptibility of target weeds. Alternatively, a small scale inoculation trial to compare the efficacy of *P. argillacea* in controlling the five different populations of *R. spectabilis* may be conducted in the future. A complementary study of the genetic diversity of *P. argillacea* could provide further information with respect to the potential risks and efficacy of *P. argillacea* as a biological control agent for *R. spectabilis*.

## 4.0. CONCLUSION

A thorough literature review of *Rubus spectabilis* was conducted to obtain a general knowledge of its origin and distribution, life history and biology, taxonomy, genetic diversity, ecological role, vegetation management using both conventional and alternative methods. *Rubus spectabilis* is a native deciduous perennial shrub, which is associated with both hardwood and softwood species specifically in BC's coastal forests where it proliferates by forming monospecific, multi-layered shrub communities with long-lived seedbanks and clonal root systems. *Rubus spectabilis* has had an economic impact in BC forestry as it is a severe competitor of newly-planted conifer seedlings in forest regeneration sites. A review of conventional methods of control, specifically, chemical and manual control, has shown inconsistent levels of control especially because damage often results in vigorous resprouting from rhizomal, basal and root buds.

One alternative means of reducing competing forest vegetation *R. spectabilis* is by biological control. This thesis investigated the potential use of several native pathogenic fungi as biological control agents for *R. spectabilis*. Naturally-occurring fungi inhabiting *R. spectabilis* were isolated from naturally-diseased *R. spectabilis* tissues and cultured on artificial media. Pathogenicity of the isolated fungal cultures was tested *in vitro* and pathogenic isolates were used to test virulence in greenhouse trials. Of the 19 isolates which were found to be moderately to highly pathogenic (causing >50% leaf area necrosis within 14 days), three *Phoma argillacea* (teleomorph *Didymella applanata*) isolates were selected for virulence testing in greenhouse trials. Inundative doses of *P. argillacea* inocula (conidia or mycelia) showed extensive necrosis of leaves and stems within 14 days. Requirements for disease development under greenhouse conditions

using *P. argillacea* includes a minimum of 18 hr of continuous leaf wetness and a mycelial inoculum concentration of 0.5% (v/v) ($3.5 \times 10^2$ CFU/mL). To fulfill requirements for developing a biological control agent, a small host range test involving non-target conifer seedlings and a commercial variety of red raspberry (var 'Meeker') was performed. Visual assessment of three economically-important conifer species, Douglas-fir, westerm hemlock and western red cedar, showed seedling tolerance to *P. argillacea*. Even though *P. argillacea* caused initial damage on red raspberry, it failed to cause the typical cane blight seen on red raspberries infected with *D. applanata*.

Another factor involved in the development of a biological control agent is the suitability of the target for biological control. The use of genetic markers is especially important when studying genetic diversity of clonal plants where the genetically effective size of a population cannot be determined from counting the number of ramets or individuals present. Hence, what appears to be a large population may in fact be small in terms of genotypes, and thus some of the factors of concern in populations of sexually reproducing plants with small numbers of individuals could also be important in the conservation of clonal plants (Esselman et al. 1999). To assess the suitability of *R. spectabilis* as a candidate for biological control, its genetic diversity was examined using RAPD-PCR. The RAPD loci used in this study represent a minimum level of diversity due to their dominant nature; however, the RAPD markers were able to show that within populations salmonberry is very diverse and that among populations salmonberry has relatively low population differentiation suggesting high gene flow. The results of this study correspond to published literature on genetic differentiation of other woody perennial species (Hamrick et al. 1992). The level of genetic differentiation between

populations was not significantly related to geographic range. This may be due to high gene flow between populations, wind patterns among populations, seed dispersal among populations or the relatively small distances between the populations sampled. Increasing the sampling range from within British Columbia to other areas such as southern California, Oregon, Washington or Alaska may have shown that *R. spectabilis* population differentiation occurs over a wider geographic scale.

It is hoped that results from this study can provide future research on how to enhance the efficacy of *P. argillacea* as a biological control agent for *R. spectabilis*, specifically in determining a suitable formulation to bypass the leaf wetness or dew period requirement of the fungus. This will ensure survival of *P. argillacea* during inundative application in the field. Several adjuvants have been used to enhance bioherbicidal efficacy of the biological control agents under field conditions (Neumann and Boland 1999; Rosskopf et al. 2000; Shabana et al. 1997) and may provide sufficient nutrition and protection to *P. argillacea.* This thesis was mainly focused on studying the environmental requirements for successful infection of *R. spectabilis* by *P. argillacea* under controlled conditions. Future experiments should test the efficacy of *P. argillacea* in controlling *R. spectabilis* under field conditions and conduct a genetic diversity of *P. argillacea,* which will complement the RAPD study on *R. spectabilis*. If the genetic diversity of *P. argillacea* will be investigated in the future, other genetic markers in addition to RAPDs should be considered such as codominant microsatellites or AFLPs, both having been used successfully to study other systems. It would also be interesting to develop species-specific markers for *P. argillacea* to monitor its environmental fate for risk assessment studies using conventional or real-time PCR.

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