

# **Investigations into a Biological Control Strategy for Lodgepole Pine Dwarf Mistletoe**

By

Tod Donald Ramsfield

B.Sc. University of Victoria, 1994  
M.Sc. Simon Fraser University, 1998

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

In

THE FACULTY OF GRADUATE STUDIES  
(Faculty of Forestry, Department of Forest Sciences)

We accept this thesis as conforming  
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 2002

© Tod Ramsfield, 2002

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of FOREST SCIENCES

The University of British Columbia  
Vancouver, Canada

Date MAY 28, 2002

## Abstract

An inundative biological control strategy for lodgepole pine dwarf mistletoe (*Arceuthobium americanum*) parasitizing lodgepole pine (*Pinus contorta* var. *contorta*) in British Columbia was investigated in this study. Of 36 collection sites in British Columbia and Alberta, *Caliciopsis arceuthobii* was observed at 8 sites and *Colletotrichum gloeosporioides* was collected from 23 sites and 187 isolates were recovered in culture. It was decided to focus on *C. gloeosporioides* because it damaged all parts of male and female *A. americanum* infections, grew readily in culture, produced abundant inoculum in culture and its distribution coincided with the range of *A. americanum* that was sampled in this study. An isolate of *C. gloeosporioides* was selected based on growth characteristics and formulated using the 'Stabileze' method for inoculation of *A. americanum* in a field trial. Two months after inoculation, the average disease rating of *A. americanum* infections treated with *C. gloeosporioides* was significantly higher than the controls. One year after inoculation, the average number of fruit present on *A. americanum* swellings that were treated with *C. gloeosporioides* was reduced, but the difference between the treatments and controls was not significant. The effect of *C. gloeosporioides* on the endophytic system of *A. americanum* was determined through culturing and histopathological examination. *Colletotrichum gloeosporioides* was cultured from the basal cup region but not from woody tissues. No fungal hyphae were observed within the endophytic tissues of *A. americanum*; however, two different types of hyphae were observed in the outer dead bark and on the bark surface. Analysis of the distribution of *C. gloeosporioides* within the canopy of lodgepole pine suggested that the presence of *C. gloeosporioides* was not related to crown position; under natural conditions, all *A. americanum* was susceptible to *C. gloeosporioides*. A study designed to follow *C. arceuthobii* infection of *A. americanum* over time found that the fungus caused an average fruit reduction of 57% each year over the first three years, and a predicted reduction of 39% in the fourth year of the study and that the fungus was able to naturally infect disease free *A. americanum*. The maximum biocontrol treatment periodicity required for prevention of fruit production was determined to be 3 years, based on the interval between shoot removal and fruit production.

## Table of Contents

<b>Abstract.....</b>	<b>ii</b>
<b>Table of Contents .....</b>	<b>iii</b>
<b>List of Tables .....</b>	<b>xi</b>
<b>List of Figures.....</b>	<b>xiii</b>
<b>Acknowledgements .....</b>	<b>xviii</b>
<b>Chapter 1: Literature Review.....</b>	<b>1</b>
1.1 Background .....	1
1.2 Dwarf mistletoes in British Columbia .....	1
1.2.1 <i>Arceuthobium americanum</i> .....	1
1.2.2 <i>Arceuthobium tsugense</i> .....	2
1.2.3 <i>Arceuthobium laricis</i> .....	3
1.2.4 <i>Arceuthobium douglasii</i> .....	4
1.3 Life cycle of <i>A. americanum</i> .....	4
1.4 Effects of dwarf mistletoe infection.....	6
1.5 Dwarf mistletoes as agents of biodiversity .....	8
1.6 Silvicultural control of dwarf mistletoe .....	9
1.7 Biological control.....	12
1.8 Examples of inundative biological control agents .....	14
1.9 Past dwarf mistletoe biological control studies .....	17

1.10 Fungi that infect <i>Arceuthobium americanum</i> .....	19
1.10.1 <i>Caliciopsis arceuthobii</i> .....	19
1.10.2 <i>Cylindrocarpon gillii</i> .....	20
1.10.3 <i>Colletotrichum gloeosporioides</i> .....	21
1.10.4 Resin Disease .....	22
1.10.5 Associations with rust fungi .....	22
1.11 Research objectives .....	23

## **Chapter 2 – Development of a biological control strategy for**

### ***Arceuthobium americanum*, Part I: Discovery and Development ..... 25**

2.1 Introduction .....	25
2.2 Materials and Methods .....	25
2.2.1 Collection of diseased <i>A. americanum</i> and hyperparasite isolation .....	25
2.2.2 Growth characteristics of <i>Colletotrichum gloeosporioides</i> .....	27
2.2.3 Sodium alginate – kaolin clay formulation .....	28
2.2.4 Inoculation of <i>Arceuthobium tsugense</i> with sodium alginate formulated <i>Colletotrichum gloeosporioides</i> under greenhouse conditions. ....	30
2.3 Results .....	32
2.3.1 Collection of fungal hyperparasites of <i>A. americanum</i> . ....	32
2.3.2 Growth characteristics of <i>Colletotrichum gloeosporioides</i> .....	41
2.3.3 Formulation .....	46
2.3.4 Inoculation of <i>Arceuthobium tsugense</i> with sodium alginate formulated <i>Colletotrichum gloeosporioides</i> under greenhouse conditions. ....	46
2.4 Discussion .....	47

2.4.1 Collection of diseased <i>A. americanum</i> and hyperparasite isolation .....	47
2.4.2 Growth characteristics of <i>Colletotrichum gloeosporioides</i> .....	49
2.4.3 Formulation .....	50
2.4.4 Inoculation of <i>Arceuthobium tsugense</i> with <i>Colletotrichum gloeosporioides</i> under greenhouse conditions. ....	51

### **Chapter 3 – Development of a biological control strategy for**

#### ***Arceuthobium americanum*, Part II: Field trial..... 53**

3.1 Introduction.....	53
3.2 Materials and Methods.....	55
3.2.1 'Stabilize' formulation .....	55
3.2.2 Site description.....	56
3.2.3 Experimental design.....	56
3.2.4 Pre-treatment assessment .....	58
3.2.5 Treatment application dates .....	59
3.2.6 Treatment assessment .....	60
3.2.7 Sampling at one year .....	60
3.2.8 Statistical analysis .....	61
3.3 Results.....	62
3.3.1 'Stabileze' formulation.....	62
3.3.2 Establishment of <i>C. gloeosporioides</i> .....	62
3.3.3 Cut shoot inoculation – Treatment 2 .....	63
3.3.4 Dead replicates one year following inoculation. ....	64
3.3.5 Effect of <i>C. gloeosporioides</i> on <i>A. americanum</i> fruit production.....	64

3.3.6 Number of female flowers .....	66
3.3.7 Effect of <i>C. gloeosporioides</i> on <i>A. americanum</i> shoot height.....	67
3.3.8 Effect of <i>C. gloeosporioides</i> on the number of <i>A. americanum</i> shoots.....	67
3.3.9 Effect of <i>C. gloeosporioides</i> on <i>A. americanum</i> bud production.....	68
3.3.10 Analysis of disease rating .....	69
3.3.11 Vigour rating analysis.....	71
3.3.12 Effect of increased humidity.....	72
3.3.13 Sampling in 2001 .....	72
3.4 Discussion .....	73

## **Chapter 4 – Cultural and histopathological examination of the infection of the endophytic system of *Arceuthobium americanum* by**

### ***Colletotrichum gloeosporioides*..... 77**

4.1 Introduction.....	77
4.2 Materials and Methods.....	80
4.2.1 <i>A. americanum</i> naturally infected with <i>C. gloeosporioides</i> .....	80
4.2.2 Artificial infection of <i>A. americanum</i> with <i>C. gloeosporioides</i> .....	81
4.2.3 Paraffin embedding.....	82
4.2.4 Staining .....	83
4.2.5 Observation and photomicrography.....	83
4.3 Results.....	83
4.3.1 Culturing and field observation of <i>C. gloeosporioides</i> inoculated <i>A. americanum</i> .....	83
4.3.1.1 <i>A. americanum</i> naturally infected with <i>C. gloeosporioides</i> .....	83

4.3.1.2 Artificial infection of <i>A. americanum</i> with <i>C. gloeosporioides</i> .....	84
4.3.2 Histopathological examination.....	86
4.3.2.1 <i>C. gloeosporioides</i> infected <i>A. americanum</i> shoots.....	86
4.3.2.2 <i>C. gloeosporioides</i> infected <i>A. americanum</i> parasitizing <i>P. contorta</i> var. <i>latifolia</i> branch tissue.....	89
4.3.2.3 <i>C. gloeosporioides</i> inoculated <i>A. americanum</i> . ....	94
4.4 Discussion.....	94
4.4.1 Culturing.....	94
4.4.2 Histopathology.....	95
4.4.3 Potential <i>C. gloeosporioides</i> exclusion mechanism.....	96
4.4.4 Conclusion.....	96
4.4.5 Future research.....	97

## **Chapter 5: Incidence of *Colletotrichum gloeosporioides* on**

### ***Arceuthobium americanum* in the crown of lodgepole pine ..... 98**

5.1 Introduction.....	98
5.2 Materials and Methods.....	99
5.2.1 Stand and tree selection.....	99
5.2.2 Data collection.....	99
5.2.2.1 Trees.....	99
5.2.2.2 <i>Arceuthobium americanum</i> .....	99
5.2.3 Statistical Analysis.....	100
5.3 Results.....	100
5.3.1 Individual trees .....	100

5.3.2 Crown position.....	102
5.3.3 Canopy position.....	103
5.3.4 Canopy aspect.....	105
5.3.5 <i>A. americanum</i> sex and the presence of <i>C. gloeosporioides</i> .....	106
5.3.6 <i>A. americanum</i> horizontal canopy position.....	106
5.4 Discussion.....	107

## **Chapter 6 – Infection of *Arceuthobium americanum* by *Caliciopsis***

<b><i>arceuthobii</i>.....</b>	<b>111</b>
6.1 Introduction.....	111
6.2 Materials and Methods.....	111
6.2.1 Stand characteristics.....	111
6.2.2 <i>Arceuthobium americanum</i> selection.....	112
6.2.3 Data collection and analysis.....	112
6.3 Results.....	114
6.3.1 <i>Caliciopsis arceuthobii</i> infection of <i>Arceuthobium americanum</i> .....	114
6.3.2 Fruit production on <i>A. americanum</i> .....	115
6.3.3 Movement in the stand.....	117
6.4 Discussion.....	118

## **Chapter 7 – *Arceuthobium americanum* response to shoot removal....**

7.1 Introduction.....	121
7.2 Materials and Methods.....	122
7.2.1 Partial shoot removal experiment.....	123

7.2.2 Statistical analysis .....	124
7.2.3 Total shoot removal .....	124
7.3 Results.....	126
7.3.1 Partial shoot removal experiment.....	126
7.3.1.1 Effects of treatment on growth of the endophytic system .....	126
7.3.1.2 New shoot production .....	127
7.3.1.3 Fruit production .....	129
7.3.1.4 Cut shoots.....	130
7.3.1.5 Control .....	131
7.3.2 Total shoot removal .....	131
7.3.2.1 Fruit production .....	133
7.3.2.2 Endophytic system growth.....	135
7.3.2.3 Shoot production.....	135
7.4 Discussion.....	136
7.4.1 Endophytic system growth.....	136
7.4.2 New shoot production.....	137
7.4.3 Fruit production.....	137
7.4.4 Response of shoots to dieback from the tip.....	138
7.4.5 Shoot characteristics over time.....	138
7.4.6 Biological control treatment periodicity.....	139
7.4.7 Conclusions.....	140
<b>Chapter 8 – Conclusions.....</b>	<b>141</b>
<b>Literature Cited .....</b>	<b>145</b>

<b>Appendix 1 – Temperature on inoculation day (July 23, 2000) and immediately following.....</b>	<b>157</b>
<b>Appendix 2 – Relative humidity on inoculation day and immediately following.....</b>	<b>158</b>
<b>Appendix 3 – Histological staining methods .....</b>	<b>159</b>
<b>Appendix 4 – ANOVA tables for canopy study, Chapter 6.....</b>	<b>162</b>
<b>Appendix 5 – Diagrammatic representation of the lifecycle of <i>Caliciopsis arceuthobii</i> and fruit production by <i>Arceuthobium americanum</i> .....</b>	<b>163</b>
<b>Appendix 6 – Presence of <i>Caliciopsis arceuthobii</i> on <i>Arceuthobium americanum</i>.....</b>	<b>165</b>

## List of Tables

Table 2-1. Treatments formulated in sodium alginate – kaolin clay and number of replicates applied to <i>A. tsugense</i> infecting <i>T. heterophylla</i> .....	32
Table 2-2. Sites visited during the collection phase of the research program and the number of <i>Colletotrichum gloeosporioides</i> isolates collected from, and where <i>Caliciopsis arceuthobii</i> was observed on, <i>Arceuthobium americanum</i> parasitizing <i>Pinus contorta</i> var. <i>latifolia</i> . ....	34
Table 3-1. Treatment description and number of replicates per treatment in the field trial designed to test the efficacy of <i>C. gloeosporioides</i> as a biological control agent for <i>A. americanum</i> . ....	57
Table 3-2. Vigour rating classes developed to classify individual <i>A. americanum</i> infections.....	58
Table 3-3. Number of living dwarf <i>A. americanum</i> infections prior to treatment and one year following treatment application. ....	64
Table 3-4. Distribution of the number of fruit present on <i>A. americanum</i> infections in 2000 and 2001. ....	66
Table 3-5. Number of <i>A. americanum</i> infections of each disease rating (DR) for all treatments two months after inoculation. ....	70
Table 3-6. Number of <i>A. americanum</i> infections of each disease rating (DR) for all treatments one year after inoculation. ....	71
Table 4-1. Recovery of fungi in culture from <i>A. americanum</i> subjected to treatment 2 and 3 during the field trial. A total of 10 replicates of the treatment and 1 of the control were randomly selected. ....	86
Table 5-1. Characteristics of individual trees surveyed in this study. ....	101
Table 5-2. Infection of <i>A. americanum</i> by <i>C. gloeosporioides</i> at different tree crown thirds. ....	102
Table 6-1. Average reduction in fruit production caused by <i>Caliciopsis arceuthobii</i> infection of <i>Arceuthobium americanum</i> . ....	116
Table 7-1. Treatments applied at the partial shoot experiment and number of replicates. ....	124
Table 7-2. Mortality two years after treatment. ....	126

Table 7-3. Partial shoot removal experiment. Average number of fruit present on female <i>A. americanum</i> infections of each treatment at every assessment and average number of flowers present at the final assessment. ....	129
Table 7-4. Average response of each variable measured up to 3 years following treatment application at both experimental locations. All measurements are recorded in centimeters. Trt = treatment, Ck = control. ....	132
Table 7-5. Number of control replicates of <i>A. americanum</i> in each category to record consecutive crops of fruit at Canal Flats and Lytton. ....	135
Table appendix 4-1. Two-way analysis of variance to compare trees and canopy thirds. ....	162
Table appendix 4-2. Two-way analysis of variance to compare trees and canopy aspect. ....	162
Table appendix 6-1. Number of <i>Caliciopsis arceuthobii</i> infected <i>Arceuthobium americanum</i> flowers observed at the Knife Creek Block of the Alex Fraser Research Forest, near 150 Mile House, British Columbia. ....	165

## List of Figures

- Figure 2-1. Laboratory apparatus for sodium alginate – kaolin clay formulation of *C. gloeosporioides*. 1. Magnetic stir plate; 2. sodium alginate – kaolin clay + *C. gloeosporioides*; 3. peristaltic pump, 4. Pasteur pipettes; 5. 0.25M CaCl<sub>2</sub> solution and pellets..... 29
- Figure 2-2. Geographic location of: A. *Arceuthobium americanum* collection sites, B. Sites where *Colletotrichum gloeosporioides* isolates were collected and, C. Where *Caliciopsis arceuthobii* was observed during this study..... 37
- Figure 2-3. *Colletotrichum gloeosporioides* infection of *Arceuthobium americanum* and morphological characteristics. 1. Lesion on shoot, scale bar represents 1 mm, E = epidermis. 2. Acervuli exuding conidia (C), scale bar represents 1 mm. 3. Conidia (C) production on diseased fruit, scale bar represents 1 mm. 4. Conidia (C), scale bar represents 100 µm. 5. Appressoria (A), scale bar represents 100 µm. 6. Culture on MEA..... 39
- Figure 2-4. Perithecia (P) of *Caliciopsis arceuthobii* infecting female flowers of *Arceuthobium americanum*. Scale bar represents 1 mm..... 41
- Figure 2-5. *C. gloeosporioides* colony diameter (mm) after 12 days incubation at temperatures ranging from 0°C to 35°C. Points plotted are the mean +/- standard error of the mean. .... 42
- Figure 2-6. Colony growth. Increase in colony diameter of *C. gloeosporioides* incubated at 20°C over 20 days. Mean +/- standard error of the mean..... 43
- Figure 2-7. Percent conidial germination of *C. gloeosporioides* isolates at temperatures ranging from 0°C to 35°C..... 44
- Figure 2-8. Germ tube elongation at temperatures ranging from 0°C to 35°C after 18 hours incubation. Mean +/- standard error of the mean..... 45
- Figure 3-1. Typical *A. americanum* infections of each vigour rating. 1. Vigour rating 1. 2. Vigour rating 2. 3. Vigour rating 3. 4. Vigour rating 4. 5. Vigour rating 5... 59
- Figure 3-2. Infection of *A. americanum* by *C. gloeosporioides* as a result of application of treatment 1, 'Stabileze' formulated *C. gloeosporioides* sprayed to run-off (DR = disease rating, VR = vigour rating). This replicate was not enclosed with a polyethylene bag. A. Pre-inoculation, DR = 0, VR = 5. B. One month post-

inoculation, DR = 1, VR = 5. C, Two months post-inoculation, DR = 2, VR = 5. D. One year post-inoculation, DR = 5, VR = 0. ....	63
Figure 3-3. Average number of fruit per female <i>A. americanum</i> infection of each treatment prior to treatment application in 2000 and one year following application in 2001. Treatment 2 excluded from 2001 ANOVA analysis. Mean +/- standard error of the mean. ....	65
Figure 3-4. Average number of female flowers present one year after inoculation. Treatment 2 excluded from ANOVA. Mean +/- standard error of the mean. ....	66
Figure 3-5. Average maximum shoot height (mm) of <i>A. americanum</i> swellings prior to treatment application in 2000 and one year following application in 2001. Treatment 2 excluded from 2001 ANOVA. Mean +/- standard error of the mean. ....	67
Figure 3-6. Average number of shoots per <i>A. americanum</i> infection prior to treatment application in 2000 and following inoculation in 2001. Treatment 2 excluded from ANOVA in 2001. Mean +/- standard error of the mean. ....	68
Figure 3-7. Average number of buds (shoots <5mm high) present on <i>A. americanum</i> infections prior to treatment in 2000 and one year following treatment in 2001. Treatment 2 included in ANOVA in 2001. Mean +/- standard error of the mean... ..	69
Figure 3-8. Average disease rating of <i>A. americanum</i> infections two months and one year following inoculation. Mean +/- standard error of the mean. ....	70
Figure 3-9. Average vigour rating of <i>A. americanum</i> prior to treatment application and at one year following application. Treatment 2 excluded from ANOVA in 2001. Mean +/- standard error of the mean.....	72
Figure 4-1. <i>Colletotrichum gloeosporioides</i> infected <i>Arceuthobium americanum</i> shoots. Safranin – Picro=analine stain. Scale bars represent 100 µm. 1. Acervulus (A) of <i>C. gloeosporioides</i> producing conidia (C). Longitudinal section. 2. Acervulus of <i>C. gloeosporioides</i> . Cross section. Note: hyphae present in the center of the shoot at the xylem core. Epidermis (E), xylem (X). 3. Acervulus of <i>C. gloeosporioides</i> prior to breaking through the epidermal layer. Acervuli is integrated with host mesophyll cells. Hypha (H). 4. Longitudinal section of diseased shoot with intercellular hyphae (H) of <i>C. gloeosporioides</i> . Cells are disrupted and not organized. 5. Longitudinal section of healthy <i>A. americanum</i> shoot tissue with	

healthy, ordered mesophyll cells. Epidermis (E). 6. Diseased and healthy <i>A. americanum</i> tissue, showing the extent of necrosis. Acervuli (A) are present on the upper and lower shoot surfaces, close to the node (node is not in micrograph). Epidermis (E). .....	87	
Figure 4-2. Transverse sections of healthy and <i>C. gloeosporioides</i> infected <i>A. americanum</i> tissues on and in lodgepole pine bark. Scale bars represents 100 $\mu$ m. 1. Spores (S) of melanized hyphae, on the surface of the bark? 2. Melanized hyphae (MH) on bark surface. 3. Melanized hyphae outside of bark tissue. 4. Melanized hyphae (MH) penetrating into the outer bark tissue. 5. Putative intercellular <i>C. gloeosporioides</i> hyphae (Cg H) present near bark surface. 6. <i>C. gloeosporioides</i> (Cg H) and melanized hyphae (MH) present in the outer bark tissue of lodgepole pine. 7. <i>A. americanum</i> shoot base with <i>C. gloeosporioides</i> hyphae present between shoot base and bark (arrow). 8. <i>C. gloeosporioides</i> hyphae (Cg H) in region shown in plate G. ....		90
Figure 4-3. Xylem cells in various endophytic tissues of <i>Arceuthobium americanum</i> . Pair of micrographs in transmitted light and birefringence to show xylem cells. Scale bar represents 100 $\mu$ m. ....		92
1, 2. Long section of sinker in host xylem. Micrograph is oriented horizontally. X=host xylem, BP=borderd pit, RT=ray trachied. ....	92	
3, 4. Cross section of sinker crossing the vascular cambium. S=sinker, VC=vascular cambium, P=phloem, X=xylem. ....	92	
5, 6. Cross section of a radial cortical strand in a longitudinal section of bark. X= <i>A. americanum</i> xylem.....	92	
7, 8. Longitudinal section of a cortical strand in a longitudinal section of bark. X= <i>A. americanum</i> xylem.....	92	
Figure 5-1. Percent infection of <i>A. americanum</i> by <i>C. gloeosporioides</i> in different tree crown thirds. Mean +/- standard error of the mean. ....		103
Figure 5-2. Percent infection of <i>A. americanum</i> by <i>C. gloeosporioides</i> at different locations in the stand canopy. Mean +/- standard error of the mean. ....		104
Figure 5-3. Distribution of <i>A. americanum</i> within the canopy. Numbers above bars represent the number of trees having crowns within the interval. ....		105

Figure 5-4. Percent infection of <i>A. americanum</i> by <i>C. gloeosporioides</i> at different aspects. Mean +/- standard error of the mean .....	106
Figure 5-5. Distribution of <i>C. gloeosporioides</i> infected and uninfected <i>A. americanum</i> on the host branch. Values close to 0 represent infections close to the main stem; values close to 1 represent infections close to the branch tip. ....	107
Figure 6-1. <i>Arceuthobium americanum</i> infected by <i>Caliciopsis arceuthobii</i> . Photo taken August 17, 2001 at the Knife Creek block of the Alex Fraser Research Forest, near 150 Mile House, British Columbia. 1. <i>Caliciopsis arceuthobii</i> perithecia present on <i>A. americanum</i> flower infected in 2001. 2. <i>Arceuthobium americanum</i> flower that escaped infection in 2001. 3. Maturing fruit of <i>A. americanum</i> that escaped infection in 2000. 4. <i>Arceuthobium americanum</i> flower that was infected by <i>C. arceuthobii</i> in 2000. ....	115
Figure 6-2. Scatter to compare the number of <i>C. arceuthobii</i> infected <i>A. americanum</i> flowers at year x with the number of infected flowers at year x+1. In the 1999 vs 2000 plot, 6 data points are located at the origin, while in the plot of 2000 vs 2001, 2 data points are at the origin. ....	117
Figure 7-1. Change in <i>A. americanum</i> swelling diameter and length over the course of the partial shoot removal experiment. Treatments pooled. Mean +/- standard error of the mean.....	127
Figure 7-2. Bud production one and two years after treatment. Male and female infections pooled. Mean number of buds per <i>A. americanum</i> infection +/- standard error of the mean. ....	128
Figure 7-3. Average number of fruit per shoot over the course of the experiment for every treatment. Mean +/- standard error of the mean.....	130
Figure 7-4. Observation of control <i>A. americanum</i> infections. Number of shoots and maximum shoot length of male and female infections pooled. Mean +/- standard error of the mean for all plots.....	131
Figure appendix 1-1. Temperature on inoculation day and following. Near Lytton, British Columbia. Maximum temperature on inoculation day was 22°C at 3:19 PM. ....	157

Figure appendix 1-2. Temperature at inoculation site from July 30 to August 9, 2001. Near Lytton, British Columbia. ....	157
Figure appendix 2-1. Relative humidity on inoculation day and immediately following. Minimum relative humidity at inoculation was 46.6% at 3:19 PM. Near Lytton, British Columbia. ....	158
Figure appendix 2-2. Relative humidity at inoculation site from July 30 to August 9, 2001. Near Lytton, British Columbia.....	158
Figure appendix 5-1. Timing of ascospore release and new perithecia production by <i>Caliciopsis arceuthobii</i> infecting <i>Arceuthobium americanum</i> . ....	163
Figure appendix 5-2. Fruit production on <i>Arceuthobium americanum</i> . ....	164

## Acknowledgements

Many people and organizations have provided support to me throughout the course of the research conducted for this degree and I want to thank them all individually because without their support, this research could not have been completed.

My supervisory committee provided input throughout the research to insure that my research stayed focused and on track. My supervisor, Dr. Bart van der Kamp, was always readily available to me and provided me with many challenging questions that made me carefully consider all aspects of this research. He treated me honestly and fairly and always considered my best interests; I am honored to have been one of his students. My research supervisor, Dr. Simon Shamoun, provided much more than the materials and supplies necessary for me to conduct my research; he made me feel at home in his laboratory and I value our friendship. Dr. Will Hintz always kept his door open to me and provided me with encouragement and advice throughout my degree program, which I very much appreciate. Dr. Steve Mitchell provided the silvicultural expertise necessary to keep the development of a biological control agent relevant to the forest industry. My committee was composed of individuals who shared their enthusiasm and expertise without hesitation, and I truly thank them for making my graduate experience enjoyable.

The research involved with this degree had a very large field component and the forest health personnel of the British Columbia Ministry of Forests district offices in Invermere and Lillooet were particularly helpful. Mr. Emile Begin of the Invermere forest district allowed me to establish a research plot and insured that the plot was preserved throughout the course of the experiment. The help of Mr. Ed Senger of the Lillooet forest district was greatly appreciated. He came on collection trips with me, provided research plots and made us feel at home during our stay in Lillooet during the summers of 2000 and 2001. Ms. Claire Trethewey of the UBC Alex Fraser Research forest toured me through the forest and allowed me to establish a field trial within the forest. I appreciate her time and enthusiasm.

Dr. and Mrs. Dick Smith welcomed me into their home and allowed me to stay with them while I was traveling through BC during 1998 and 1999. Dick spent time with me collecting diseased *A. americanum* and his expertise was very valuable.

Preparation of samples and staining for the histopathological study described in Chapter 3 was conducted in Ms. Lesley Manning's laboratory. Her advice, as well of the help of Mr. Terry Holmes and Mr. Garry Jensen is greatly appreciated.

Funding for this research was provided by a GREAT award from the Science Council of British Columbia, MycoLogic Inc., and Forest Renewal British Columbia.

My lab mates Shannon, Peter, Carmen, Jennifer, Grace, Rob, Anna-Mary, Brad, Lea, Sue, Chris and Cheryl provided encouragement and friendship that made the hours spent together very enjoyable.

This work could not have been completed without the constant support of my family. Mom, Dad and Leanne, and my in-laws Peter, Pat and Christina have always believed in me and supported me, no matter what. My wife and best friend Theresa, and Abby and Ethan have endured some stressful times, but they have always loved me and stood beside me. We traveled this road together and my accomplishment is due, in a large part, to their unconditional love.

## Chapter 1: Literature Review

### 1.1 Background

Dwarf mistletoes of the genus *Arceuthobium* (Viscaceae) are obligate parasitic flowering plants of conifers within the families Pinaceae and Cupressaceae; they rely upon the host for support, mineral nutrients, a portion of their required carbon compounds, water and possibly other growth factors. Thirty-four New World species and eight Old World species are presently recognized. In North America, the greatest species diversity is located within northwestern Mexico and the western United States where twenty-eight of the thirty-four New World species are present. Five species of dwarf mistletoe have ranges that extend into Canada, *A. americanum* Nuttall ex Engelmann in Gray, *A. tsugense* (Rosendahl) G.N. Jones, *A. laricis* (Piper) St. John, *A. douglasii* Engelmann and *A. pusillum* Peck. Of the five species in Canada, all but *A. pusillum* are present in British Columbia (BC) (Hawksworth and Wiens, 1996).

### 1.2 Dwarf mistletoes in British Columbia

Dwarf mistletoe host specificity has been categorized as “principal”, “secondary”, “occasional” and “rare” and tree species that are not parasitized are classified as “immune”. These categories are based on the percent infection of host species inside a 6-meter radius plot centered on a heavily parasitized tree. In heavily infected older stands, a host is considered to be a principal host if greater than 90% of the individuals of a species within the plot are parasitized. In areas where the principal host is at least 80% infected, secondary hosts are 50% to 90% parasitized and occasional hosts are 5% to 50% parasitized. A host is classified as a rare host if less than 5% of individuals of a species are parasitized (Hawksworth and Wiens, 1972).

#### 1.2.1 *Arceuthobium americanum*

*Arceuthobium americanum*, lodgepole pine dwarf mistletoe, is the most widely distributed species within BC. It occurs east of the Coast Mountain Range to beyond the Alberta / British Columbia border and from the border with the United States to as far north as 57° latitude. The most heavily infected stands of lodgepole pine occur in the region south of Spillimacheen in the Columbia River Valley and in the area north of

Clinton to Prince George, extending westward to Anahim Lake in the Chilcotin (Baranyay and Smith, 1972). The principal host is lodgepole pine (*Pinus contorta* Dougl. ex Loud. var *latifolia* Engelm.), ponderosa pine (*Pinus ponderosa* P. Laws. Ex C. Laws.) is a secondary host and occasional hosts include white spruce (*Picea glauca* (Moench) Voss), Engelmann spruce (*Picea engelmannii* Parry ex Engelm) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco). Immune species include grand fir (*Abies grandis* (Dougl. ex D. Don) Lindl.) and mountain hemlock (*Tsuga mertensiana* (Bong.) Carrière). Aerial shoots have verticillate branching (whorled) with height of 5 to 9 (max. 30) cm and colour ranging from yellow to green. Flowering occurs from March to June (peak in May) with seed release from July to October (peak in late August and early September), approximately 16 months after pollination (Hawksworth and Wiens, 1996).

### **1.2.2 *Arceuthobium tsugense***

*Arceuthobium tsugense*, western hemlock dwarf mistletoe, is restricted to the west side of the Coast Mountain Range; including Vancouver Island and the Queen Charlotte Islands. The most heavily infected stands are located on the Queen Charlotte Islands, northern Vancouver Island, Texada Island, and near Prince Rupert and Vancouver (Baranyay and Smith, 1972). Two subspecies of *A. tsugense* are recognized in British Columbia: *A. tsugense* (Rosendahl) G.N. Jones subsp. *tsugense* and *A. tsugense* (Rosendahl) G.N. Jones subsp. *mertensianae* Hawksworth and Nickrent.

*Arceuthobium tsugense* subsp. *tsugense* has been broken down into two races, the western hemlock race and the shore pine race depending upon principal host association. Western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) is the principal host of the western hemlock race and grand fir and lodgepole pine are occasional hosts. Rare hosts include Engelmann spruce, western white pine (*Pinus monticola* Dougl. ex D. Don), Douglas fir and mountain hemlock (Hawksworth and Wiens, 1996). The shore pine race of *A. tsugense* subsp. *tsugense* occurs principally on shore pine (*Pinus contorta* Dougl. ex Loud. var. *contorta*) and is morphologically very similar to the western hemlock race. The race distinction is based upon host relationships as determined by inoculation trials (Smith and Wass, 1979).

Mountain hemlock dwarf mistletoe, *A. tsugense* subsp. *mertensianae*, has a limited range in BC; herbarium specimens present in the forest pathology herbarium at the Pacific Forestry Centre were collected from North Vancouver, West Vancouver and Port Alberni. The primary hosts are mountain hemlock, amabilis fir (*Abies amabilis* (Dougl. ex Loud) Dougl. ex J. Forbes) and subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.). The secondary host is whitebark pine (*Pinus albicaulis* Engelm.) and western white pine is an occasional host, while grand fir, lodgepole pine and western hemlock are rare hosts (Hawksworth and Wiens, 1996).

*Arceuthobium tsugense* shoots are flabellately (fan-like) branched and green to reddish in colour. Shoot height of *A. tsugense* subsp. *tsugense* ranges from 3 to 13 cm, while the shoots of *A. tsugense* subsp. *mertensianae* are typically shorter. Flowering and seed dispersal of the two subspecies are slightly different. Both subspecies flower from July to October, but subsp. *tsugense* peaks 1 to 2 weeks earlier, while seed dispersal occurs from August to December in subsp. *tsugense* and from August to November in subsp. *mertensianae*. Fruit maturation requires 13 to 14 months in subsp. *tsugense* and 12 to 13 months in subsp. *mertensianae* (Hawksworth and Wiens, 1996).

### 1.2.3 *Arceuthobium laricis*

The range of *A. laricis*, larch dwarf mistletoe, is restricted to southeastern BC in a triangle bounded by Osoyoos, St. Leon (Upper Arrow Lake) and Moyie. Heavily infected stands are present near Trail and the Creston-Kimberley area (Baranyay and Smith, 1972). Western larch (*Larix occidentalis* Nutt.) and mountain hemlock are principal hosts, lodgepole pine is a secondary host and occasional hosts include grand fir, Engelmann spruce, western white pine and whitebark pine (*Pinus albicaulis* Engelm.). The shoots of *A. laricis* average 4 (max. 6) cm, are flabellately branched and are dark purple. Flowering occurs from June to September (peak from mid-July to August) and seed dispersal occurs from July to October (peak in September), thus fruit require 13 to 14 months for maturation (Hawksworth and Wiens, 1996).

#### **1.2.4 *Arceuthobium douglasii***

*Arceuthobium douglasii*, Douglas-fir dwarf mistletoe, is widely distributed in the United States and Mexico, but is limited in BC to the Okanagan and Similkameen valleys and the Creston area. Isolated infected stands are located near Lytton, Sicamous and Rossland. *Arceuthobium douglasii* is not present in the coastal forests of BC (Baranyay and Smith, 1972). The principal host is Douglas-fir and no secondary hosts are present. Occasional hosts that occur in BC are grand fir and Engelmann spruce. The shoots of *A. douglasii* are olive green in colour, average 2 (max. 8) cm and have flabellate branching. Flowering peaks in April and May, but ranges from February to July, while seed dispersal occurs from late August to late September, thus fruit maturation requires 17 to 18 months (Hawksworth and Wiens, 1996).

#### **1.3 Life cycle of *A. americanum***

Dwarf mistletoes are dioecious, obligate parasites, requiring a living host to complete their life cycle. Initiation of new dwarf mistletoe infection begins when a mature seed is cast from the aerial shoot of a female dwarf mistletoe plant onto susceptible host tissue in the fall. Dwarf mistletoe seeds are dispersed using a hydrostatic discharge mechanism (Hinds et al., 1963) that propels the seed at speeds up to 27 meters per second (Hinds and Hawksworth, 1965). The maximum recorded seed dispersal distance of *A. americanum* was 13.7 meters (Muir, 1970 In Hawksworth and Johnson, 1989). The seed is coated with a sticky substance called viscin, which serves to adhere the seed to any object that it lands upon (Mathiasen, 1996). Viscin is a hygroscopic compound that swells after rain and lubricates the seed, allowing the seed to slide to the needle base where it adheres to the twig when the viscin dries (Hawksworth and Wiens, 1996). Not all seeds are successfully relocated to the needle base; if the needle is pointing down, the seed will slide off of the needle onto foliage below or through to the ground (Shaw and Loopstra, 1991). Germination of the seed, indicated by radicle emergence, occurs in the spring following seed dispersal and successful infection normally occurs on branches less than five years old (Hawksworth and Wiens, 1996), likely as a result of increased bark thickness of older branches (Sproule, 1996). After germination, when the radicle contacts the needle base or some other obstruction, a

holdfast structure is formed which contains a penetration wedge. The penetration wedge forces into the host tissue and initiates the formation of the root-like endophytic system consisting of sinkers in the xylem and cortical strands in the inner bark (Hunt et al., 1996). The development of the endophytic system results in the formation of a swelling on the host branch at the point of infection.

An incubation period of 2-8 years precedes shoot production (Hawksworth and Wiens, 1996). Shoot production is initiated by meristematic cells in the outer cortical strand that push the buds through the bark to the branch surface (Cohen, 1954). Flowering begins the year after shoot emergence and fruit mature over 16 months after pollination to be released from late August to early September. Individual shoots can produce multiple crops of flowers and fruit in successive years (Hawksworth and Wiens, 1996). Completion of the life cycle of *A. americanum*, from seed dispersal to fruit maturation, averages 6 years (Hawksworth and Johnson, 1989).

The endophytic system of *A. americanum* consists of radially oriented sinkers that become embedded in the xylem as the vascular cambium divides to add xylem tissue and strands that are longitudinally and tangentially oriented in the cortex and phloem (Hunt et al., 1996). To remain connected to the strands, the sinkers must divide in conjunction with the host cambium (Srivastava and Esau, 1961). Elongation of the strands occurs by transverse division of an apical cell to create a new row of segments that divide longitudinally. The new cells then undergo anticlinal and periclinal divisions to create longer strands of increased diameter (Bhandari and Nanda, 1970).

Intensification is the process by which dwarf mistletoe infection is increased in abundance and distribution within the stand. The rate of intensification is influenced by stand density, host growth rate and disease severity; the rate of intensification decreases over time as the stand reaches crown closure or dwarf mistletoe reaches maximum levels (Geils and Mathiasen, 1990).

Long distance introduction of dwarf mistletoe into new stands occurs via birds and small mammals that vector the seeds. Satellite infections, infections that have arisen at distances greater than could be achieved by explosive seed dispersal, and infections in the tops of otherwise uninfected trees are presumed to be the result of seeds that have

adhered to the feathers of birds or the fur of small mammals and have been deposited on a susceptible host (Hawksworth and Wiens, 1996).

#### **1.4 Effects of dwarf mistletoe infection**

The most obvious symptom of dwarf mistletoe infection is stimulation of the host to produce witches' brooms, which are composed of dense masses of host branches; however, every dwarf mistletoe infection does not result in the development of witches' brooms, many infections remain localized. The production of witches' brooms by diseased trees can cause drastic changes in the canopy structure of the stand (Mathiasen, 1996). Witches' brooms increase the potential for stand clearing fires because the lush foliage of the witches' broom acts as ladder fuel to bridge fire from the understory to the overstory (Anonymous, 1995). Two types of witches' brooms are formed: systemic (isophasic) brooms and non-systemic (anisophasic) brooms. In systemic brooms, the growth of the endophytic system occurs at the same rate as the growth of the cambial and apical portions of the host branch, resulting in dwarf mistletoe shoots that are scattered along the length of the branch or at each branch node. *Arceuthobium americanum* and *Arceuthobium douglasii* produce systemic witches' brooms. In non-systemic witches' brooms, the endophytic system remains localized; *Arceuthobium tsugense* and *Arceuthobium laricis* form non-systemic brooms (Kuijt, 1960).

The formation of witches' brooms is hypothesized to be induced by an imbalance of plant growth regulators in the infected host tissue. Cytokinin and indole acetic acid (IAA) concentration of infected branches was higher and abscisic acid (ABA) was lower than uninfected branches of the same age of black spruce (*Picea mariana* (Mill) B.S.P.) infected by *A. pusillum* (Livingston et al., 1984). The exact mode of action of these hormones on witches' broom development is not clear. Increased cytokinins and IAA, or one hormone independently, may disrupt the apical dominance of the meristem of infected branches, allowing a proliferation of lateral twigs if dormant meristems are present. Alternatively, decreased ABA may have the same affect. It is also not known if the hormones are produced by the dwarf mistletoe or if the dwarf mistletoe stimulates the host to change the production of these compounds (Mathiasen, 1996).

The physiological effects of dwarf mistletoe infection on the host with respect to wood quality, volume production and carbohydrate metabolism have been studied. When lumber from *Arceuthobium abietinum* Engelman ex Munz f. sp. *concoloris* Hawksworth & Wiens infected and uninfected white fir from California was graded, a greater proportion of high grade boards were cut from logs that derived from dwarf mistletoe infected trees. The authors concluded that either dwarf mistletoe does not affect lumber quality or that the quality control procedures utilized were unable to detect differences (Wilcox et al., 1973). An alternative explanation for this observation is that the most affected wood is the outer sapwood, which is removed during slabbing (Hawksworth and Johnson, 1989). Dwarf mistletoe infection of the host tree has been found to affect wood quality throughout the entirety of infected tree, not only in regions that are directly parasitized (Piirto et al. 1974). When microscopic examination of dwarf mistletoe infected wood was conducted to compare infected and non-infected wood from diseased trees and wood from healthy trees, it was found that the percentage of latewood was lower in the diseased trees than the controls. Microfibril angle was increased in infected wood (leading to increased shrinkage of dried lumber), alcohol-benzene extractive content was three times higher while tracheid length, modulus of elasticity, modulus of rupture and work to proportional limit were all reduced (Piirto et al. 1974). It was suggested by Piirto et al. (1974) that these microscopic differences may not be noticed at the sawmill, as was indicated by Wilcox et al. (1973). In a study designed to compare lumber yields and visual grades of dwarf mistletoe infected lodgepole pine and healthy lodgepole pine, Dobie and Britneff (1975) found no differences in lumber grade and recovery; however, dwarf mistletoe infected trees were older and shorter than non-infected trees, suggesting that dwarf mistletoe infection severely retards volume production. Dwarf mistletoe infections on the main stem may also lead to breakage due to failure of the wood in the infected region.

It has been hypothesized that dwarf mistletoe causes a reduction in volume by acting as a carbon sink. Carbohydrate production by dwarf mistletoe through photosynthesis is not enough to supply the entire plant; the host supplies the balance of carbohydrates (Leonard and Hull, 1965). The increased carbohydrate production induced by the dwarf mistletoe results in the infected branch acting as a carbon sink (Clark and

Bonga, 1970) causing carbon to be diverted from other portions of the tree and resulting in decreased volume (Broshot and Tinnin, 1986).

Volume losses to dwarf mistletoe have been estimated at 3.8 million cubic meters annually in western Canada and 11.3 million cubic meters in the western United States. The economic impact of this volume loss is difficult to calculate exactly, but it has been suggested that it totals several billion dollars annually (Hawksworth and Wiens, 1996). The impact of dwarf mistletoes in British Columbia is ranked in the top three forest diseases in British Columbia (Nevill and Winston, 1994).

Dwarf mistletoe infection of young trees results in high mortality while infection of older trees results in decreased needle length, decreased length of needle bearing branches, decreased needle surface area and a decrease in the total number of needles. As dwarf mistletoe severity increases, diameter and height growth decrease resulting in reduced volume production. In some host-parasite combinations mortality is increased by dwarf mistletoe infection (summarized from Hawksworth and Wiens, 1996).

### **1.5 Dwarf mistletoes as agents of biodiversity**

The presence of dwarf mistletoe in a stand being managed for timber production has a negative economic impact; however, where timber production is not the primary management objective, the presence of dwarf mistletoe has positive effects.

Several insects have been observed to feed on dwarf mistletoe shoots. Most feeding occurs by insects that feed incidentally and opportunistically; however, insects that are obligate feeders on dwarf mistletoe shoots do exist. Obligate feeders include: larvae of *Mitoura spinetorum* (Lycaenidae), the thicket hairstreak butterfly, *Dasypyga alternosqualmella* (Pyalidae), *Filatima natalis* (Gelechiidae), *Neoborella tumida* (Miridae), and *Pityophthorus arceuthobii* (Scolytidae). Several mites and spiders have also been associated with dwarf mistletoes; however, their role has not been thoroughly explored (Hawksworth and Wiens, 1996).

Bird species have been observed to utilize dwarf mistletoe fruits as a food source and to utilize witches' brooms as nesting sites. The relationship between bird habitat and dwarf mistletoe was studied in Colorado within ponderosa pine that was parasitized by *A. vaginatum* subsp. *cryptopodum* (Engelmann) Hawksworth and Wiens. It was found that

bird species diversity and density was correlated with dwarf mistletoe abundance. Dwarf mistletoe infection often results in snag creation and the density of cavity nesting birds was found to increase with increasing dwarf mistletoe infection (Hawksworth and Wiens, 1996).

Small mammals also feed on dwarf mistletoe shoots, as well as dwarf mistletoe infected branches; however, no mammals are obligate feeders on dwarf mistletoes. The red squirrel has been observed to eat the living bark tissues of dwarf mistletoe swellings and to nip off small dwarf mistletoe infected branches and carry the branches to seed caches (Baranyay, 1968). In a study of wildlife usage of *A. douglasii* induced witches' brooms, Parks et al. (1999) found that the nests that were observed in the witches' brooms were all mammal nests and they hypothesized that the presence of mammals prevented bird nesting. Use of dwarf mistletoe stands by large mammals has been studied and mule deer and elk in Colorado preferred dwarf mistletoe infected stands (Bennetts et al., 1991 in Hawksworth and Wiens, 1996). Parks et al. (1999) observed that the area under large brooms was used as a resting site for large mammals because the brooms provided shelter from rain and snow.

### **1.6 Silvicultural control of dwarf mistletoe**

Stand management objectives dictate which, if any, dwarf mistletoe control strategies will be utilized. Dwarf mistletoe causes reduced volume production, loss of wood quality and increased mortality, which are not desirable in stands managed for timber production. Selection of the proper management regime must be carefully considered because forest harvesting and regeneration can limit or enhance dwarf mistletoe spread and intensification (Anonymous, 1995). Since dwarf mistletoe is an obligate parasite, it can be destroyed by killing infected parts of the host tree or all infected trees. This characteristic allows dwarf mistletoe management to be integrated into normal forest management practices, resulting in control of dwarf mistletoe with little extra cost (Baranyay and Smith, 1972), but it does result in limiting the silvicultural options. Other characteristics of dwarf mistletoes that make them amenable to silvicultural control include: host specificity, long life cycles, slow spread within stands

and easy diagnosis due to the presence of witches' brooms (Hawksworth and Johnson, 1989).

Factors that effect dwarf mistletoe spread and intensification within a stand include stand density, host vigour, tree age, stand composition and stand history. Dwarf mistletoe spread occurs more rapidly in open stands because as density increases, seed interception increases, slowing spread. Stand density is related to tree vigour, in that open stands result in greater light penetration and less competition for nutrients. This results in trees with fuller crowns, therefore increasing seed interception. Additionally, increased host vigour results in increased dwarf mistletoe vigour. Tree age is important in dwarf mistletoe spread because young trees are small and are more likely to escape infection. Mixed stands have lower rates of spread because non-hosts serve to intercept seeds and reduce infection on susceptible species. Stand history, especially fire, affects the dwarf mistletoe presence in a stand. If a dwarf mistletoe infected stand is not burned during a fire, it serves as an inoculum source to invade the adjacent replacement stand. Witches' brooms act as ladder fuel to carry fire from a low intensity ground fire up into the crown, resulting in a stand clearing fire. This has the effect of removing dwarf mistletoe from the stand, but also perpetuating seral species that are susceptible to dwarf mistletoe (Parmeter, 1978).

Prevention of dwarf mistletoe establishment in regenerating stands is a much more effective management tool than removal of infected trees from infected stands after stand establishment (Hawksworth and Johnson, 1989). Cutblock design provides an opportunity to prevent establishment of dwarf mistletoe in the new stand. Knowledge of the distribution of dwarf mistletoe within the stand prior to cutting is essential so that cut-block boundaries do not intersect the dwarf mistletoe infected areas. Natural boundaries to dwarf mistletoe spread, such as roads, utility rights-of-way and resistant species can be utilized as cut-block boundaries to prevent spread of dwarf mistletoe into the new stand from the edge. Where dwarf mistletoe free boundaries are not possible, large clear-cut blocks reduce the ratio of perimeter to edge and reduce the proportion of the area invaded by dwarf mistletoe. In such situations, narrow strips should be avoided when designing cut blocks because infection of the new stand from the stand edge will occur rapidly (Baranyay and Smith, 1972).

If alternative silvicultural systems, such as shelterwood, variable retention, or a seed tree system are to be utilized, steps can be taken to avoid dwarf mistletoe infection of the regenerating stand. If the seed-tree system is being used for establishment of the new stand, the seed trees left after harvesting should be dwarf mistletoe free or lightly infected. If infected trees must be left, they should be removed or girdled as soon as seedlings are established (Anonymous, 1995). If the shelterwood system is being used, the overstory trees should be non-host trees or trees with little or no infection, and as with the seed tree system, infected residuals should be removed as soon as the stand is established (Anonymous, 1995). If the stand is being managed under an uneven-aged selection system, non-host species and trees with little or no dwarf mistletoe infection should be left as residuals (Anonymous, 1995).

The host specificity of dwarf mistletoe can be exploited when establishing a new stand in dwarf mistletoe infected areas. Planting immune tree species at the edge of a cut block boundary will reduce ingress of the dwarf mistletoe into the stand. When the silvicultural system utilized requires retention of the overstory, planting immune tree species in the understory will reduce dwarf mistletoe in the future stand (Baranyay and Smith, 1972). Planting immune conifer species as a barrier to dwarf mistletoe infection is not always effective since susceptible species may naturally regenerate from a seed bed. The resulting mixed stand will likely allow dwarf mistletoe spread into the pure stand, especially in the case of *A. americanum* and lodgepole pine because lodgepole pine has great early height growth and rapidly overtops the non-hosts (van der Kamp, personal communication).

In British Columbia, the Forest Practices Code of 1995 legislated smaller cut-block size, increased riparian reserves and more partial cutting, reducing the options for silvicultural control of dwarf mistletoe. Riparian areas serve as refugia for dwarf mistletoe because the damp environment resists fire; increased riparian reserves may result in increased dwarf mistletoe entry into regenerating stands (van der Kamp, personal communication).

Constrained silvicultural options for dwarf mistletoe control in partial cuts has rekindled the search for alternative dwarf mistletoe management strategies. Biological control has been studied in the past; however, a suitable biological control strategy has

not been developed. Chemical control has also been investigated and almost 60 different formulations, mostly mixtures of 2,4-D or 2,4,5-T, have been tested. None were able to control the dwarf mistletoe without coincidental damage to the host and none affected the endophytic system (Hawksworth and Wiens, 1996). The only chemical that reduces the rate of spread of dwarf mistletoe is the plant growth regulator ethephon (2-chloroethyl phosphoric acid), which causes shoot abscission. The chemical does not affect the endophytic system and rapid shoot resprouting occurs following shoot removal; 52% of treated female *A. americanum* infections bore fruit after 5-years (Nicholls, 1988). Ethephon is registered by the Environmental Protection Agency in the United States (Hawksworth and Wiens, 1996) under the name Florel® (Rhone-Poulenc Ag Co), but it is not registered for forestry use in Canada.

Genetic resistance to dwarf mistletoes has been noted in the western hemlock – *A. tsugense* pathosystem (Smith et al., 1993) and in the ponderosa pine – *A. campylopodum* pathosystem (Roth, 1974). Clonal propagation of western hemlock with a range of susceptibility to *A. tsugense* allowed increased numbers of genetically identical replicates for testing. Seed germination rate did not differ significantly between clones, but two of the selected clones had low levels of infection. The resistance mechanism was not determined, however, it appeared that the penetrating structure of the dwarf mistletoe was unable to enter the host cortex (Smith et al., 1993). The mode of resistance of ponderosa pine to *A. campylopodum* is hypothesized to be a result of foliar and physiological characteristics (Roth, 1974). Although genetic resistance has been suggested, no genetically resistant seed stock is available for plantation establishment (Hawksworth and Wiens, 1996).

### **1.7 Biological control**

The biological control strategy is the deliberate use of living organisms to suppress or reduce the effects of a pest to acceptable levels (Mortensen, 1998). Advantages of biological control include specificity to the target, no persistent residue or toxicity hazards because biological control agents and their metabolites are biodegradable, a potentially sustainable effect because the biological control agent might become a persistent component of the ecosystem and, because multiple genes are

involved in pathogenesis, a low probability of resistance development (Holdenrieder and Greig, 1998; Wilson, 1969). Disadvantages of biological control include sensitivity to climatic conditions, limited shelf life and possible effects on non-target organisms, which may result in a shift in the biodiversity of the system (Holdenrieder and Greig, 1998). Furthermore, because the specificity of biological control agents limits their market potential, a limited market must pay the development and registration costs (Mathre et al., 1999). Two main approaches to biological control have been utilized: classical (or inoculative) biological control and inundative biological control (inundative biological control agents are often referred to as bioherbicides or mycoherbicides) (Mortensen, 1998).

Classical biological control assumes that the target has escaped its natural enemies, thus allowing it to proliferate. Natural enemies are sought out in other regions and introduced into areas where they are absent and the target is a problem, resulting in a reduction in the competitive ability of the target and a reduction in the target population (Mortensen, 1986). The ultimate goal of classical biological control is to establish a natural, self-regulating balance between the host and the pathogen (Hasan and Ayres, 1990). Classical biological control agents must cause severe damage to the target organism without damaging other organisms in the area of introduction. Thorough host range testing under greenhouse conditions must be conducted to ensure the safety of native, non-target, species (Mortensen, 1998).

The inundative biological control approach utilizes indigenous strains of fungi or other organisms that are well adapted to the local environment. This approach overcomes many of the barriers of classical biological control, such as spatial, temporal and environmental constraints to establishment. The advantage of the inundative biological control strategy over the classical biological control strategy is that efficacy is not reliant upon the agent being self-sustaining; long term survival of the agent is not as important because inundative biological control agents are typically re-applied in years following initial treatment (Hintz et al., 2001). The agent is released by a single, timely application of inoculum at a climatically suitable time that results in infection, disease development and eventual death of the specific host (Templeton, 1992). The release of a large

concentration of inoculum can overcome the natural constraints that limit epidemic development under natural conditions (Mortensen, 1998).

The strategy for development of inundative biological control agents consists of three major stages: (1) discovery; (2) development and; (3) deployment. During the discovery stage, the organism of interest is identified and extensive field collections of diseased samples are made. Under laboratory conditions, the pathogens present are isolated from the organism and identified. Pure cultures of the pathogens are then applied to healthy and uninfected hosts under greenhouse conditions and the disease process is monitored. Once the symptoms are described, each pathogen is re-isolated from the diseased tissue and identified. The identity of the pathogen isolated from the diseased plant must be same as that which the plant was initially inoculated with and result in the same symptoms to prove Koch's postulates (Manion, 1981), and thereby prove that the potential biological control agent is the causal agent of the disease. The development stage involves determination of conditions for optimum inoculum production, disease development, host range and evaluation of the efficacy of the pathogen as a potential biocontrol agent. During the deployment stage, mass production strategies for scale-up, formulation, regulatory, and marketing strategies for the biocontrol agent are developed (Watson and Wall, 1995). During these stages, the essential criteria of: (1) ability to produce abundant and durable inoculum in artificial culture; (2) genetic stability of the inoculum and specificity to the target organism; and (3) ability to infect and kill the host under variable environmental conditions, are all assessed (Templeton, 1982; Sands et al., 1990). Failure of the potential biological control agent to meet any of these criteria will result in the search for alternative pathogens.

### **1.8 Examples of inundative biological control agents**

Several inundative biological control agents have been commercialized for weed control in agriculture and forestry. In conifer regeneration sites, competition for light, water and nutrients from hardwood species such as red alder (*Alnus rubra* Bong.) slows the growth of regenerating trees; therefore, these hardwoods are considered weeds (Biring et al., 1996). *Chondrostereum purpureum* (Pers.:Fr) Pouzar, is a basidiomycete fungus that is able to rapidly colonize cambium and sapwood of inoculated trees (Wall,

1991), and it has been observed to control red alder and other weedy hardwood species with the same efficacy as chemical herbicide when applied to the cut stump (Shamoun et al., 1996; Harper et al., 1999; Pitt et al., 1999). This fungus has been registered under the trade name BioChon™ in the Netherlands by Koppert Biological Systems for control of American black cherry (*Prunus serotina* Erhr.) and in North America under the trade name Myco-Tech™ in Quebec and is currently being registered by MycoLogic Innovative Biologicals Inc. under the trade name Chontrol™ (Shamoun, Personal Communication). In South Africa, *Cylindrobasidium laveae* (Pers.:Fr.) Chamuris has been registered as Stumpout™ for control of *Acacia* spp. (Lennox et al., 2000). Other inundative biological control strategies for competitors of forest regeneration include *Fusarium avenaceum* (Fr.) Sacc., *Colletotrichum dematium* (Pers.) Grove and *Phomopsis* sp. for control of *Rubus* spp. (Oleskevich et al., 1998) and *C. dematium* for fireweed control (*Chamerion angustifolium* L. ssp. *angustifolium*) (Winder and Watson, 1994).

Inundative biological control of forest diseases has also been investigated. *Heterobasidion annosum* (Fr.:Fr.) Bref., the causal agent of annosus root and butt rot, infects stumps of susceptible trees via spores, colonizes the stump and is able to infect adjacent trees through root grafts. Inoculation of stumps with *Peniophora gigantea* (Fr.) Masee (syn. *Phlebiopsis gigantea* (Fr.) Jülich or *Phlebia gigantea* (Fr.) Donk or *Phanerochataete gigantea* (Fr.:Fr.) Rattan, Abdullah & Ismail) following cutting results in colonization of the stump by *P. gigantea*, preventing colonization by *H. annosum* through competitive exclusion. Several other fungi have also been utilized for *H. annosum* control and these include: *Bjerkandera adusta* (Willd.:Fr.) P. Karst., *Fomitopsis pinicola* (Sw.:Fr.) P. Karst., *Resinicium bicolour* (Albertini & Schwein.:Fr.) Parmasto, *Hypholoma* spp., *Melanotus proteus* (Kalchbr.) Singer, *Armillaria* spp., *Trichoderma* spp., and *Scytalidium* spp. (Holdenrieder and Greig, 1998; Mercer, 1988). *Peniophora gigantea* has been registered in Europe and was commercialized as RotStop™ in Finland (Shamoun, Personal Communication). Inundative biological control of *Armillaria ostoyae* (Romagn.) Herink through competitive exclusion by *Hypholoma fasciculare* (Huds. Ex. Fr.) has been investigated in British Columbia (Chapman and Xiao, 2000). Placing *H. fasciculare* in close proximity to Douglas-fir or

pine stumps resulted in colonization of the stumps although the direct impact on *A. ostoyae* has not yet been quantified.

Although examples of inundative biological control strategies for utilization in forestry scenarios exist, the majority of research on inundative biological control is targeted at weeds in agricultural systems. In China, since 1966, *C. gloeosporioides* f. sp. *cuscutae*, referred to as Luboa™, has been applied to control dodder (*Cuscuta chinensis* Lam. and *C. australis* R. br.) parasitizing soybeans (*Glycine max* (L.) Merr.) (Watson et al., 2000). Round-leaved mallow (*Malva pusilla* Sm.) is a common farmyard and garden weed that has become a problem in field crops. The fungus *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *malvae* was isolated from round-leaved mallow and tested as an inundative biological control agent (Mortensen, 1988). The isolate was registered as BioMal™ in 1992, and the potential of the agent was high (Templeton, 1992), but it was never marketed due to the high cost of inoculum production (Watson et al., 2000). The taxonomic classification of the BioMal™ isolate was reassessed, based on ribosomal DNA and the infection process, and changed to *C. orbiculare* (Berk. & mont.) von Arx (syn. *C. lagenarium* (Pass.) Ell. & Halst.) (Bailey et al., 1996). This change in classification was recently supported by studies of the infection process of the BioMal™ isolate, which was found to utilize the same infection process as *C. orbiculare* (Shen et al., 2001). Another forma specialis of *Colletotrichum gloeosporioides* that has been registered as an inundative biological control agent is *C. gloeosporioides* f. sp. *aeschynomene*, under the trade name Collego™ for the control of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P.) in rice and soybean fields (Templeton, 1992). The product was marketed from 1982 to 1992 through three successive producers and not re-registered due to low perceived market potential. Collego™ was re-registered in 1997 and approximately 5,000 hectares were treated in 1998 (Watson et al., 2000). In South Africa, *C. gloeosporioides* has been registered to control silky needlebush (*Hakea sericea* Schrader) (Watson et al., 2000). Several other inundative biological control studies have investigated the potential of *Colletotrichum*, including: control of hemp sesbania (*Sesbania exaltata* (Rydb.) ex. A.W. Hill) with *Colletotrichum truncatum* (Schw.) Andrus & Moore. (Boyette et al., 1993), control of Koster's curse (*Clidemia hirta* (L.)) in Hawaiian forests with *Colletotrichum gloeosporioides* f. sp. *clidemiae* (Norman and

Trujillo, 1995). Other species of fungi have also been tested for their efficacy as inundative biological control agents, including *Phytophthora palmivora* (Butler) Butler (registered as DeVine™) for stranglervine (*Morrenia odorata* Lindl.), *Alternaria* spp., *Cercospora rodmanii* Conway, *Fusarium* spp., *Nectria ditissima* (Tul) for red alder and *Puccinia canaliculata* (Schw.) Lagerh. (registered as Dr. Biosedge™) for yellow nutsedge (*Cyperus esculentus* L.) (Mortensen, 1998), and *Valdensinia heteroxa* Peyronel for control of salal (Shamoun et al., 2000).

Commercial success for inundative biological control agents that utilize *Colletotrichum* spp. has been limited. There are many reasons for this, including: expectation that inundative biological control would replace chemical herbicides, limited shelf-life, extended dew requirements, low fecundity, low virulence and restricted niche markets. Current research in inundative biological control is shifting away from isolating new strains of fungi to solving production, storage and efficacy problems. Future usage of inundative biological control agents will likely occur as an integrated pest management approach in association with chemical or biological compounds and silvicultural tools such as manual brushing to increase efficacy to a satisfactory level (Watson and Wall, 1995; Watson et al., 2000).

### **1.9 Past dwarf mistletoe biological control studies**

The attributes of a successful biological control agent for dwarf mistletoes were outlined by Wicker and Shaw (1968) and included: 1. distribution which coincides with that of the target pathogen; 2. ecologic amplitude sufficient to ensure persistence within its host range; 3. production of abundant inoculum for establishment of epiphytotics; 4. high infectivity; 5. high virulence and; 6. efficient mode of action for curtailing development of the target host. These criteria incorporate attributes of both classical and inundative biological control agents.

Several fungi, including *Caliciopsis arceuthobii* (Peck) Barr, *Cylindrocarpon gillii* (D.E. Ellis) Muir and *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. have been observed to infect dwarf mistletoe and many authors have suggested that these fungi be utilized as biological control agents (Muir, 1967; Parmeter et al. 1959; Wicker and Shaw, 1968). In spite of this, there is a paucity of literature describing inoculation trials.

Parmeter et al. (1959) inoculated *A. campylopodum* f. *abietinum* with conidia of *C. gloeosporioides*, Knutson and Hutchins (1979) inoculated *A. douglasii* with *Caliciopsis arceuthobii* and Ellis (1946) inoculated *A. douglasii* with *Cylindrocarpon gillii*. These inoculation studies were conducted to prove Koch's postulates; only one field trial was conducted to test the utility of a fungus, *Cylindrocarpon gillii*, as a biological control agent (Mielke, 1959).

When biological control of dwarf mistletoes was initially proposed (Ellis, 1946), and the only real attempt to establish a fungus as a biological control agent was initiated (Mielke, 1959), it was envisioned that biological control would occur following the classical biological control model. In the case of dwarf mistletoes, the dwarf mistletoe host and fungal pathogen are indigenous organisms that have co-evolved (Hawksworth and Wiens, 1996); therefore, it is not surprising that Mielke (1959) found no evidence of *C. gillii* 3 years after inoculation. Likely, there is a natural equilibrium that exists between the dwarf mistletoe and the fungal hyperparasite, analogous to the system proposed by van der Kamp and Blenis (1996) to regulate the western gall rust (*Endocronartium harknessii* (J.P. Moore) Hirat.) / hard pine pathosystem. Muir (1977), quantified the natural occurrence of *C. gloeosporioides* on *A. americanum* in Alberta, and observed a 35% infection of *A. americanum* at one site and 75% infection of *A. americanum* at another site. Muir (1977) concluded that although *C. gloeosporioides* caused a significant reduction in dwarf mistletoe shoot and fruit production, the fungus would not significantly reduce the impact of *A. americanum* because the number of new dwarf mistletoe plants was not significantly reduced by natural infection of *A. americanum* by *C. gloeosporioides*.

There are many scenarios in which inundative biological control may prove to be a useful option to the forest manager. If timber production with retained overstory is the primary stand management objective, prevention of dwarf mistletoe entry into the regenerating stand from overstory and adjacent trees using inundative biological control may allow retention of the overstory trees without the risk of dwarf mistletoe infection. Under the forest practices code of British Columbia, reduced clearcut size and increased riparian reserves will lead to increased dwarf mistletoe spread and intensification; however, inclusion of biological control in the silvicultural treatment of the stand may

reduce this spread. In areas such as parks, where large witches' brooms increase the hazard rating of trees (Hadfield et al., 2000), application of a biological control agent to prevent new dwarf mistletoe infections in trees surrounding camping or picnicking sites would prevent dwarf mistletoe spread and intensification while retaining the infected host tree.

### **1.10 Fungi that infect *Arceuthobium americanum***

The fungi that parasitize dwarf mistletoes have been termed hyperparasites because they cause disease on dwarf mistletoe, an obligate parasite. The hyperparasites described below cause disease on *A. americanum* and have been observed to regulate the dwarf mistletoe through interfering with the dwarf mistletoe life cycle by reducing seed production or shoot mortality.

#### **1.10.1 *Caliciopsis arceuthobii***

*Caliciopsis arceuthobii* (ex. *Wallrothiella arceuthobii* (Peck) Saccardo) is a highly specialized ascomycete that infects the female flowers of the spring flowering dwarf mistletoes *A. pusillum* (Peck, 1875 in Weir, 1915), *A. douglasii* (Weir, 1915), *A. americanum* (Dowding, 1931) and *A. vaginatum* sub sp. *cryptopodium* (Hawksworth and Wiens, 1996). In Canada, this fungus has been collected from *A. americanum* in British Columbia (Kuijt, 1969; Wood, 1986), Alberta and Manitoba (Dowding, 1931) and *A. pusillum* in Quebec (Pomerleau, 1942 in Kuijt, 1969), however, the Quebec population was recorded as extinct by Kuijt (1969).

The presence of *C. arceuthobii* is readily identified by the presence of 40 – 50 black perithecia on the female flower. Ascospores mature in the asci within the perithecia in March and April and are then released during the dwarf mistletoe flowering period. It is hypothesized that insects transport the ascospores to susceptible female flowers, where the ascospore germinates and infects the stigmatic tissues. Infection of the female flower prevents development of the seed and perithecia are present on *A. americanum* in the fall of the year the infection was initiated (Kuijt, 1969). The life cycle of *C. arceuthobii* therefore requires two seasons for completion (Knutson and Hutchins, 1979).

*Caliciopsis arceuthobii* has been cultured on medium that contained glucose, asparagine and 0.2% yeast extract (Parker, 1970) as well as potato dextrose agar (PDA) + 1% yeast extract (Knutson and Hutchins, 1979). On both media, the fungus grew very slowly, radial growth was 20 mm after 6 months on the glucose – asparagine media (Parker, 1970) and on PDA, a diameter of 5 mm was reached after 4 months (Knutson and Hutchins, 1979). The difference in growth rates may be attributable to the origin of *C. arceuthobii*; Parker (1970) isolated the fungus from *A. americanum* while Knutson and Hutchins (1979) isolated the fungus from *A. douglasii*. The colonies became black with age in both studies. No perithecia or asci were produced in culture (Knutson and Hutchins, 1979), although Dowding (1931) recovered “sprout cells” from surface sterilized stromata tissue. Parker (1970) suggested that these “sprout cells” were a contaminant. The presence of another fungus on the perithecia of *C. arceuthobii* has been recorded (Dowding, 1931; Kuijt, 1969; Knutson and Hutchins, 1979) and was identified as *Cladosporium* sp. in Kuijt’s (1969) study. Knutson and Hutchins (1979) identified the other fungus as *Aureobasidium pullulans* (de Bary) Arn. Kuijt (1969) observed, and Knutson and Hutchins (1979) isolated, *C. arceuthobii* from *A. americanum* and *A. douglasii*, respectively, possibly accounting for the difference in the fungal associate. Dowding (1931) believed that the fungus arising from the stomata tissue was the imperfect state of *C. arceuthobii*; however, Knutson and Hutchins (1979) did not recover conidia from stromata that arose from ascospore cultures. The imperfect state of *C. arceuthobii*, if it exists, is unknown.

#### 1.10.2 *Cylindrocarpon gillii*

*Cylindrocarpon gillii* (ex. *Septogloeum gillii* D.E. Ellis) was first described by Gill (1935) on *A. tsugense* and *A. abietinum* Engelm ex Munz in Washington and *A. cyanocarpum* (A. Nelson ex Rydberg) Coulter & Nelson, *A. microcarpum* (Engelmann) Hawksworth and Wiens and *A. abietinum* in Arizona although it was not named. Ellis (1939) observed the fungus on *A. douglasii* in Arizona and tentatively placed it in the genus *Fusarium*. Later, Ellis (1946) named the fungus as *Septogloeum gillii* and extended the host range to include *A. blumeri* A. Nelson, *A. divaricatum* Engelm and *A. campylopodum* Engelm in Gray. Gill (1952) observed *C. gillii* on *A. americanum*

in Montana. In Canada, *C. gillii* has been recorded in British Columbia on *A. americanum*, *A. douglasii* and *A. tsugense* (Wood, 1986). Muir (1973) reclassified the fungus as *Cylindrocarpon* based on conidiophore morphology and spore formation.

*Cylindrocarpon gillii* primarily infects the dwarf mistletoe shoots and occasionally the fruit. Small yellow-white spots are initially formed in the spring, which converge and erupt through the epidermal layer during the summer to expose white spore masses (Ellis, 1946). On the host, phialospores are non- to three-septate, 12-41 x 3-5 µm and in culture, phialospores are non- or one-septate, 6-14 x 3-4 µm. Phialides were 30-80 x 1-3 µm and chlamydospores of 10-14 µm diameter occurred singly or in groups of up to four and are present in older cultures but not hosts. The fungus grows slowly, reaching 5 mm diameter after 14 days (Muir, 1973).

#### 1.10.3 *Colletotrichum gloeosporioides*

Unlike the hyperparasites described above, *Colletotrichum gloeosporioides* is a pathogen that occurs on a wide variety of plant hosts and crops worldwide (Prusky et al., 2000). It was first described on *Arceuthobium abietinum* infecting red fir (*Abies magnifica* Murr.) in California (Parmeter et al., 1959). *Colletotrichum gloeosporioides* also infects *A. americanum* and *A. campylopodum* in California (Scharpf, 1964), Idaho and Washington (Wicker, 1967; Wicker and Shaw, 1968) and *A. douglasii* and *A. laricis* in Idaho and Washington (Wicker and Shaw, 1968). In Canada, it has been observed on *A. americanum* in British Columbia (Wood, 1986), Alberta and Saskatchewan (Muir, 1967). Recently, *C. gloeosporioides* was isolated from *A. tsugense* subsp. *tsugense* on Vancouver Island (Kope et al., 1997). *Colletotrichum gloeosporioides* has also been isolated from European mistletoe (*Viscum album* subsp. *typicum* Beck.) (Stojanović, 1989).

Symptoms of *Colletotrichum gloeosporioides* infection first appear as dark brown to black lesions, usually at the shoot nodes. The lesions enlarge and acervuli rupture through the epidermis and under moist conditions, salmon to cream coloured masses of conidia are exuded from the acervuli. No setae were observed in acervuli on infected shoots (Parmeter et al., 1959). Conidia are hyaline, 15-18 x 4.5-5.5 µm, single celled becoming two celled at germination. Appresoria are produced on germ tubes (Parmeter

et al., 1959). Infection of the endophytic system of *Arceuthoium* sp. has been reported (Parmeter et al., 1959; Wicker and Shaw, 1968) although the pathological affects of infection of the endophytic system have not been described. In culture, isolates of *C. gloeosporioides* from different *Arceuthobium* hosts had different growth rates, colony colour and sporulation rates but cross inoculation indicated that the isolates were not host specific (Scharpf, 1964). The sexual stage of *Colletotrichum gloeosporioides* is *Glomerella cingulata* (Stonem.) Spaulding & Schrenk (Mordue, 1971).

#### 1.10.4 Resin Disease

Resin disease is a disease syndrome of *Arceuthobium americanum* that is hypothesized to be the result of the combination of 11 different fungi, the most common being *Alternaria alternata* (Fries) Keissler and *Aureobasidium pullulans*. The disease has been found in Colorado, Idaho, Montana, Utah and Wyoming and is characterized by excessive resin exudation from the swellings, necrotic and discoloured bark, formation of a necrophylactic periderm and retention of dead, resin filled, needles. The formation of the necrophylactic periderm is hypothesized to be the mechanism of shoot death because it isolates the shoots and cortical strands from the sinkers. The authors suggest that the resin disease complex would be a good biological control method for *A. americanum* (Mark et al., 1976). It is doubtful, however, that the fungi isolated from diseased swellings are the sole cause of resin disease because the fungi implicated were endophytic fungi that were commonly isolated from healthy *A. americanum* during a survey of the mycoflora associated with *A. americanum* (Gilbert, 1984).

#### 1.10.5 Associations with rust fungi

*Arceuthobium americanum* has often been located in association with *Cronartium comandrae* Pk., a rust fungus, on lodgepole pine. An experiment was designed to investigate the possibility that *A. americanum* may be acting as a telial host of *C. comandrae*. Inoculation of *A. americanum* did not result in infection by *C. comandrae*, indicating that it is likely not a host for *C. comandrae* (Peterson, 1966).

*Peridermium bethelii* Hedgecock & Long, is very similar to *C. comandrae* and is almost always found in association with dwarf mistletoes, including *A. americanum*. The

distinguishing feature that separates these rust fungi is the morphology of the aeciospores; aeciospores of *P. bethelii* are smaller and subglobose in shape. No alternate hosts for *P. bethelii* have been observed and inoculation of comandra (the alternate host of *C. comandrae*) was unsuccessful. Field observation suggests that *P. bethelii* is a pine-to-pine rust. It is possible that *P. bethelii* represents an autoecious, microcyclic ecotype of *C. comandrae*. The exact details of the relationship between *P. bethelii* and *A. americanum* have not been established (Hawksworth et al., 1983).

### 1.11 Research objectives

The research conducted in this study was focused toward the development of an inundative biological control strategy for *Arceuthobium americanum* infecting *Pinus contorta* var. *latifolia* in British Columbia. The research milestones are outlined below.

1. To survey *A. americanum* in British Columbia and to collect and describe hyperparasitic fungi that have been reported to cause disease on *A. americanum*.
2. To select a lead isolate of the candidate hyperparasite based on mycelial growth over a range of temperatures, conidial germination over a range of temperatures and conidia production in culture in the absence of experimental units of *A. americanum* for greenhouse testing.
3. To formulate the selected lead isolate in different formulations and determine which formulation will provide the best inoculation system for a field trial. Formulation is necessary because it protects the hyperparasite against desiccation, provide nutrients, and aids in application, ultimately leading to increased efficiency.
4. To conduct a field trial to test the selected hyperparasite and assess the potential of the fungus as a biological control agent for *A. americanum*.
5. To describe the pathological effects of the selected hyperparasite on the endophytic system of *A. americanum* to elucidate the pathogenesis process of the selected hyperparasite using histopathological methods.
6. To describe the natural distribution of the hyperparasite within the canopy of *A. americanum* infected lodgepole pine trees to determine if there are any constraints to hyperparasite development on *A. americanum* at different canopy positions.

7. To conduct trials that mimic the action of a biological control agent by shoot removal to determine how the dwarf mistletoe responds, and particularly, the time required for new shoot and fruit production. These data are necessary to develop guidelines for inundative biological treatment interval required to prevent *A. americanum* seed production.

These studies were conducted to increase the understanding of the role of hyperparasitic fungi in regulating the life cycle of *A. americanum* and to investigate the potential of an inundative biological control strategy to mitigate the impact of *A. americanum* on lodgepole pine stands.

Note: In the following chapters the word “infection” has been utilized to describe both part of the pathogenesis process of *A. americanum* on lodgepole pine and hyperparasites on *A. americanum* as well as the dwarf mistletoe plant on the host, i.e. “Spread to *A. americanum* infections that escaped initial biological control application...”

## **Chapter 2 – Development of a biological control strategy for *Arceuthobium americanum*, Part I: Discovery and Development**

### **2.1 Introduction**

Prevention of dwarf mistletoe entry into newly established stands can be achieved by effective silvicultural planning (Baranyay and Smith, 1972; Van Sickle and Wegwitz, 1978; Hawksworth and Johnson, 1989). In British Columbia however, current forest practices are moving towards smaller cut block size, increased riparian reserves and increased use of partial harvesting systems that will promote dwarf mistletoe spread. The changes in forest management have led to a renewed search for alternative management strategies, such as inundative biological control, to minimize dwarf mistletoe impact on regenerating stands. The ability to interfere with the life cycle of dwarf mistletoe, and thus reduce spread, is the most important characteristic of an inundative biological control agent.

This chapter details studies that were conducted during the initial discovery and development phases of an inundative biological control approach for *Arceuthobium americanum* infecting *Pinus contorta* var. *latifolia* in British Columbia. The objectives of this research phase were to: 1. collect diseased *A. americanum* to isolate hyperparasitic fungi (pathogens of dwarf mistletoe) that may be utilized as biological control agents; 2. to study the growth characteristics of the selected hyperparasite; 3. to formulate the hyperparasite in a medium to allow long term storage and to increase efficacy and; 4. to test the inoculation system under greenhouse conditions in advance of a field trial.

### **2.2 Materials and Methods**

#### **2.2.1 Collection of diseased *A. americanum* and hyperparasite isolation**

Diseased *A. americanum* shoots, fruit and swellings were collected in the province of British Columbia during the summer of 1998. Forest health officers of the British Columbia Ministry of Forests directed me to *A. americanum* infected lodgepole pine stands within their districts. Lodgepole pine stands in the area bounded by Quesnel in the north, the Canada – United States border in the south, the British Columbia –

Alberta border in the East and Coast Mountains in the west were visited and inspected for the presence of *A. americanum*. Additionally, *A. americanum* in a stand of lodgepole pine near Nordegg, Alberta and a stand of Jack pine (*Pinus banksiana*) near Bruderheim, Alberta were inspected for diseased *A. americanum*.

If dwarf mistletoe was located in the stand, the presence of fungal disease on the shoots was assessed and diseased samples were collected for later fungal isolation in the laboratory. Dead *A. americanum* swellings on dead branches were not collected in order to avoid isolation of saprophytic fungi; swellings that were collected had either obvious signs of infection or they had shoots with necrotic and healthy tissue.

At the laboratory, diseased *A. americanum* shoots were surface sterilized by placing the diseased material in 95% ethanol for 3 minutes, then 10% sodium hypochlorite for 3 minutes, followed by 3 washes in sterile distilled water for 3 minutes per wash. The surface sterilized pieces were then transferred to potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) and incubated at 20°C. If hyphae emerged from the dwarf mistletoe material, a piece of media containing hyphal tips was subcultured onto a new PDA plate and incubated at 20°C. If characteristic *Colletotrichum gloeosporioides* lesions were present on the diseased shoot, the lesions were wetted with sterile distilled water for five minutes and then a sterile loop was used to streak the conidia onto a PDA plate. Plates were then incubated at 20°C and observed for the production of single conidia colonies. Single conidia colonies were transferred to new PDA plates and the culture identity was confirmed based on morphological characteristics. For long term storage, cultures were placed on 1.5% malt extract agar slants and stored at 5°C.

Attempts to isolate *Caliciopsis arceuthobii* from parasitized female flowers were conducted as follows: 1. perithecia were aseptically removed from the female flower and attached to the lid of a petri plate so that ascospores could be cast onto media below; 2. perithecia were crushed in sterile distilled water and streaked onto media; 3. perithecia were surface sterilized in ethanol and sodium hypochlorite and placed directly on media. In all cases, the fungus was placed on glucose-asparagine media (glucose, 30.0 g, asparagine, 1.0 g, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g, KH<sub>2</sub>PO<sub>4</sub>, 1.5 g, water, 1 L) modified by the addition of 0.2% yeast extract and 1.5% agar (Parker, 1970).

### **2.2.2 Growth characteristics of *Colletotrichum gloeosporioides*.**

The growth response of *C. gloeosporioides* at temperatures ranging from 0°C to 35°C, increasing in 5°C increments, was determined. The rate of linear mycelial growth, percent conidial germination and germ tube length at each temperature were measured. To measure mycelial growth rate at various temperatures, isolate PFC-4277 collected at Logan Lake, BC, isolate PFC-4278 collected from Nordegg, AB, and isolates PFC-4493 and PFC-4501 from Canal Flats, BC were selected. To measure mycelial growth, a mycelial plug was placed in the center of a PDA plate and incubated at each temperature in the dark. There were five replicates of each isolate at each temperature. Every other day, the plates were placed under a dissecting microscope and the leading edge of the hyphal front was drawn on the plate using a permanent fine point marker. The experiment was concluded after 21 days incubation, when the hyphal margin of the fastest growing isolate contacted the edge of the petri plate, at which time the growth marks were measured. Growth on each plate was measured by averaging two diameters at right angles to each other.

Conidia for the germination studies were produced in 250 ml Erlenmeyer flasks by inoculating 20 g of millet grain that was soaked in 20 ml H<sub>2</sub>O and autoclaved for 20 minutes with 2 mycelial plugs of each isolate. The inoculated grain was allowed to incubate for 2 weeks at room temperature with frequent agitation. There were 2 replicate flasks per isolate. The conidia were washed from the grain by adding 100 ml of sterile dH<sub>2</sub>O to each flask and placing the flask on a rotary shaker at 225 rpm for 30 minutes. The contents of each flask were then poured through sterile cheesecloth into sterile beakers. The filtered conidia were placed in multiple 50 ml centrifuge tubes, centrifuged at 2500 *xg* for 10 minutes, the supernatant was decanted and the conidia were resuspended in 2 ml of sterile distilled water by vortexing. The contents of all centrifuge tubes from each flask were then combined so that all of the conidia of each replicate flask were in one centrifuge tube. The tubes were centrifuged as before, the supernatant was decanted and the conidia resuspended in 2 ml of sterile dH<sub>2</sub>O by vortexing. The two tubes for each isolate, each tube representing the conidia of one flask, were combined, centrifuged as before, the supernatant was decanted and the conidia were finally resuspended in 10 ml of sterile dH<sub>2</sub>O. Conidial production of each isolate was quantified

by making a  $10^{-2}$  dilution of the stock suspension and then using a hemacytometer to determine the concentration of conidia in the stock suspension.

Conidia were diluted to  $1 \times 10^5$  conidia per ml and then 500  $\mu$ l was spread onto a 1.5% water agar plate for the germination and germ tube length study. One plate per isolate was incubated at temperatures ranging from 0°C to 35°C, increasing in 5°C intervals, for 18 hours in the dark. All isolates were incubated in the same incubators over the same period of time to insure uniform incubation conditions. To insure that each plate had 18 hours growth prior to measurement, all plates were placed at 4°C after 18 hours incubation to slow growth to a negligible amount. Conidial germination was recorded as percent germination of approximately 300 conidia surveyed on each plate. Germ tube length was calculated by averaging the germ tube length of 10 randomly selected germinated conidia on each plate. Conidia were selected for measurement if the direction of germ tube elongation matched the plane of the ocular micrometer in the microscope eyepiece. The germ tube was considered germinated if germ tube length was greater than the diameter of the conidia.

### **2.2.3 Sodium alginate – kaolin clay formulation**

The formulation method utilized was encapsulation of conidia and mycelial fragments within sodium alginate pellets based on the protocol of Walker and Connick (1983). Isolate PFC-4277 was grown in 250 ml of modified Richard's media, containing 50 g commercial sucrose, 10 g  $\text{KNO}_3$ , 5g  $\text{KH}_2\text{PO}_4$ , 2.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02g  $\text{FeCl}_3$ , 150 ml V-8 juice and distilled water to 1 litre. The pH of the medium was adjusted to 6.0 using 50% NaOH (Templeton, 1992). The media was inoculated with mycelial fragments and placed on a rotary shaker at 125 rpm for 1 week at room temperature. After incubation, the culture was poured into an autoclaved blender and pulsed for approximately 30 seconds. After blending, the culture was examined under the microscope for damage to conidia and conidia were quantified using the hemacytometer. The total number of conidia harvested was calculated to be  $3.1 \times 10^9$  resulting in a final concentration of conidia in the formulated product of  $2.2 \times 10^6$  conidia per ml. The contents of the blender flask, as well as 125 mg of streptomycin sulfate (Fisher Scientific, Fair Lawn, NJ), were then transferred into a sterile solution of 16.625 g sodium alginate

(BDH, Toronto, ON) and 125 g kaolin clay (Sigma, St. Louis, MO) suspended in 1 litre of distilled water. The suspension had been autoclaved the previous day and placed on a stirrer over night to insure that the kaolin was well mixed and that the suspension was cool prior to inoculation. After addition of the fungal culture and antibiotic, the suspension was stirred for 45 minutes for complete homogenization. To form individual pellets, the suspension was pumped out of the flask using a peristaltic pump and dropped through Pasteur pipettes into a 0.25 M solution of  $\text{CaCl}_2$  (Figure 2-1). The chemical reaction between sodium alginate and  $\text{CaCl}_2$  resulted in the formation of pellets 3 mm in diameter as soon as the droplet entered the  $\text{CaCl}_2$  solution. The pellets were removed from the  $\text{CaCl}_2$  solution using a strainer and washed with sterile distilled water to remove excess  $\text{CaCl}_2$  and then placed on foil covered trays and allowed to air dry.

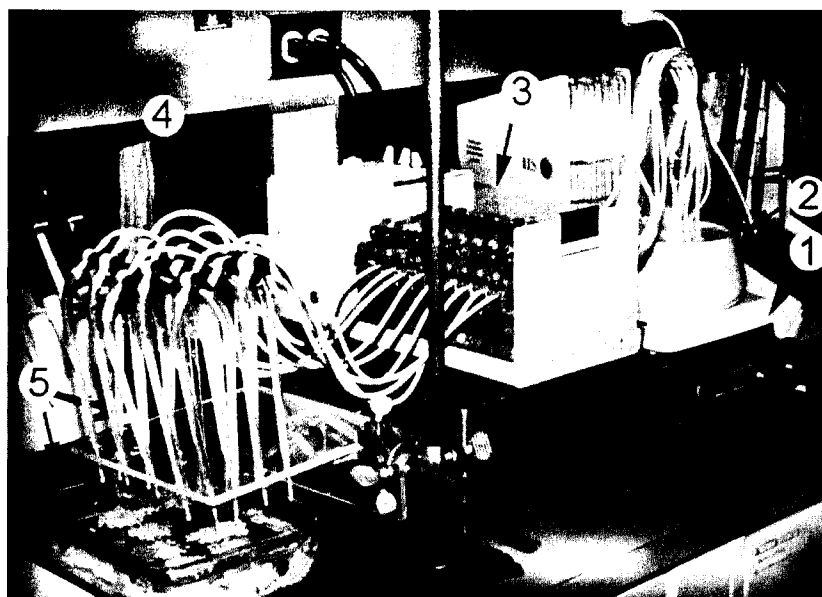


Figure 2-1. Laboratory apparatus for sodium alginate – kaolin clay formulation of *C. gloeosporioides*. 1. Magnetic stir plate; 2. sodium alginate – kaolin clay + *C. gloeosporioides*; 3. peristaltic pump, 4. Pasteur pipettes; 5. 0.25M  $\text{CaCl}_2$  solution and pellets.

*Colletotrichum gloeosporioides* isolate PFC-2329 collected from *A. tsugense* by Dr. Simon Shamoun (Canadian Forest Service, Pacific Forestry Centre) was also formulated at a final concentration of  $1.15 \times 10^5$  conidia per ml. A sterile control was formulated by adding sterile modified Richard's media and streptomycin sulfate to the

kaolin / sodium alginate mix, followed by dropping the suspension into  $\text{CaCl}_2$  and air drying.

To test the viability of the fungus after formulation, fresh and dried pellets were placed on PDA and incubated at 20°C. Viability was determined by the emergence of fungal hyphae from the pellet. Dried pellets were also ground to a fine powder using a mortar and pestle and placed on PDA to assess emergence from the ground formulation. Dried pellets were stored at 4°C until required. Viability of pellets stored at room temperature and 4°C was verified by plating out on PDA after 108 days storage.

The number of conidia within each pellet of the PFC-4277 formulation was calculated by dropping 1 ml of the solution into  $\text{CaCl}_2$  and the resultant number of pellets counted. A total of 26 pellets were formed from 1 ml of solution, therefore each pellet contained 85,500 conidia. To achieve a concentration of  $1.0 \times 10^6$  conidia per ml, approximately 12 pellets were used for each ml of inoculum, or 0.055 g of dried pellets per ml. To suspend the pellets for application, and to provide protection from desiccation (Boyette et al., 1993), a series of corn oil and water emulsions were made, with oil concentration of 100%, 75%, 50%, 25%, 20%, 15%, 10% and 5% by volume (Egley and Boyette, 1995). Pellets were removed from storage at 4°C and ground to a fine powder using a mortar and pestle. To each 1 ml emulsion, 0.055 g of powdered pellets were added. The powder was suspended by vortexing and four 100 µl drops were placed on each PDA plate, with 3 replicate plates per emulsion. The plates were then incubated at 20°C and the emergence of the fungus was observed.

#### **2.2.4 Inoculation of *Arceuthobium tsugense* with sodium alginate formulated *Colletotrichum gloeosporioides* under greenhouse conditions.**

A small-scale greenhouse trial was conducted to test the efficacy of the sodium alginate formulated *C. gloeosporioides*. Experimental material was limited to western hemlock (*Tsuga heterophylla*) seedlings that were inoculated with *Arceuthobium tsugense* 37 months prior to the trial. A total of 16 *A. tsugense* infections were present on 12 trees and prior to inoculation, the number of shoots and maximum shoot length of each *A. tsugense* swelling were measured. Six treatments were applied, with 5 treatments having 3 replicates per treatment and 1 treatment with 1 replicate as outlined in table 2-1.

Two isolates of *C. gloeosporioides* were formulated and applied; PFC-2329 was isolated from *A. tsugense* and PFC-4277 was isolated from *A. americanum*. As an alternative to corn oil emulsion, a carrier solution containing 2% sucrose and 0.5% gelatin (Trujillo et al., 1994) was utilized. A sterile sucrose – gelatin solution was placed in a spray atomizer and treatment 1 was applied by spraying the shoots to run off. Treatments 2, 4 and 5 were performed by first spraying the *A. tsugense* infection with the sucrose – gelatin solution to run off followed by sprinkling the aerial shoots and swelling with the sodium alginate – kaolin clay formulation that had been ground to a fine powder with a mortar and pestle. After sprinkling the formulated *C. gloeosporioides* onto the dwarf mistletoe, the inoculated *A. tsugense* was misted with the sucrose – gelatin solution to hydrate the formulation. Treatment 3 was applied by spraying a suspension of PFC-4277 conidia at  $1 \times 10^6$  conidia per ml suspended in the sucrose – gelatin solution to run off with a spray atomizer. Treatment 6 was applied by cutting the dwarf mistletoe shoots 5 mm from the shoot base followed by immediate application of 10 to 20  $\mu$ l of the suspension used for treatment 3. After treatment application, a sample of each treatment was placed on PDA and incubated in the dark at 20°C to check the viability of the formulated conidia. For 60 hours following inoculation, the humidity in the greenhouse chamber was raised to near 100% with a humidifier and continual soaking of the cement floor.

Table 2-1. Treatments formulated in sodium alginate – kaolin clay and number of replicates applied to *A. tsugense* infecting *T. heterophylla*.

Treatment #	Treatment description	# replicates	Tree #
1	Control 1: sucrose – gelatin only	3	5
2	Control 2: sucrose – gelatin and sterile sodium alginate pellets	3	12, 15, 20
3	Conidia of PFC-4277 at $1.0 \times 10^6$ / ml	3	6, 16
4	Formulation of PFC-4277	3	9, 11
5	Formulation of PFC-2329	3	2, 7
6	Shoots cut 5mm from base and conidia of PFC-4277 at $1.0 \times 10^6$ / ml applied to cut shoots	1	3

A disease rating system was developed that was based on the percent area of the dwarf mistletoe aerial shoots covered by lesions and necrotic tissue to quantify the amount of disease. A value from 0 to 5 was assigned based on the percent necrosis as follows: 0 = no necrosis, 1 = 1% to 25%, 2 = 26% to 50%, 3 = 51% to 75%, 4 = 76% to 100%, 5 = dead. The term of the experiment was 55 days and disease rating was assessed every other day for the first 23 days and then weekly during the remaining 22 days. At the end of the experimental period, the number of shoots and maximum shoot height were recorded.

## 2.3 Results

### 2.3.1 Collection of fungal hyperparasites of *A. americanum*.

Diseased *A. americanum* was collected from lodgepole pine stands within southern interior of the province of British Columbia and in Nordegg, Alberta during the summer of 1998. A stand of jack pine near Bruderheim, Alberta was also sampled. A total of 36 sites were visited (Table 2-2, Figure 2-2 panel A) and 504 fungal isolates were collected and lodged in Dr. Simon Shamoun's culture collection at the Pacific Forestry Centre. Of the 504 isolates collected, 187 were isolates of *Colletotrichum gloeosporioides*. The remainder of the fungi isolated included *Cladosporium* sp. and

*Sclerophoma pithyophila*, as well as other fungi that were not identified. *Colletotrichum gloeosporioides* was collected throughout the region sampled, except from Bruderheim, AB (Figure 2-2 panel B). The distribution of *Caliciopsis arceuthobii* in British Columbia was limited (Figure 2-2 panel C), and no isolates of the fungus were recovered. *Cylindrocarpon gillii* symptoms were not observed, nor was the fungus isolated in culture from any of the sites that were surveyed in 1998.

Table 2-2. Sites visited during the collection phase of the research program and the number of *Colletotrichum gloeosporioides* isolates collected from, and where *Caliciopsis arceuthobii* was observed on, *Arceuthobium americanum* parasitizing *Pinus contorta* var. *latifolia*.

Site	Location <sup>†</sup>	GPS (site)	Date (m/d/y)	Number of <i>C. gloeosporioides</i>	<i>C. arceuthobii</i> observed (yes/no)
1	150 Mile House	52° 02' 52"N 121° 48' 39"W	5/13/98	5	Yes
2	150 Mile House	52° 02' 19"N 121° 46' 54"W	5/13/98	0	Yes
3	Riske Creek	51° 56' 00"N 122° 47' 07"W	5/14/98	17	No
4	Riske Creek	51° 58' 10"N 122° 47' 33"W	5/14/98	0	No
5	Riske Creek	51° 59' 41"N 122° 50' 30"W	5/14/98	6	No
6	100 Mile House	51° 30' 40"N 121° 22' 23"W	5/16/98	0	No
7	Beaverdell	49° 22' 51"N 119° 06' 06"W	6/11/98	2	No
8	Beaverdell	49° 29' 54"N 119° 07' 35"W	6/11/98	6	Yes
9	Beaverdell	49° 30' 59"N 119° 06' 02"W	6/11/98	0	No
10	Greenwood	49° 04' 49"N 118° 35' 25"W	6/12/98	2	No
11	Greenwood	49° 04' 05"N 118° 36' 21"W	6/12/98	0	No
12	Greenwood	49° 09' 29"N 118° 37' 30"W	6/12/98	5	No

Site	Location <sup>†</sup>	GPS (site)	Date (m/d/y)	Number of <i>C. gloeosporioides</i>	<i>C. arceuthobii</i> observed (yes/no)
13	Cranbrook	49° 26' 01"N 115° 54' 00"W	6/13/98	0	No
14	Cranbrook	49° 22' 15"N 116° 04' 19"W	6/14/98	0	No
15	Cranbrook	49° 23' 19"N 115° 35' 33"W	6/14/98	16	No
16	Bull River	49° 29' 17"N 115° 26' 58"W	6/15/98	0	No
17	Invermere	50° 27' 48"N 116° 14' 06"W	6/16/98	13	No
18	Radium Hot Springs	50° 39' 34"N 116° 13' 32"W	6/16/98	8	Yes
19	Radium Hot Springs	50° 30' 28"N 115° 40' 49"W	6/16/98	0	No
20	Canal Flats	50° 12' 50"N 116° 02' 20"W	6/16/98	4	No
21	Moyie	49° 16' 06"N 115° 36' 45"W	6/17/98	0	No
22	Logan Lake	50° 29' 34"N 120° 29' 43"W	7/5/98	12	No
23	Logan Lake	50° 29' 06"N 120° 52' 34"W	7/5/98	22	No
24	Riske Creek	51° 46' 43"N 122° 39' 10"W	7/7/98	1	Yes
25	Riske Creek	51° 43' 53"N 122° 58' 11"W	7/7/98	5	No
26	Riske Creek	51° 43' 49"N 123° 02' 05"W	7/7/98	3	No
27	Big Lake	52° 27' 34"N	7/8/98	0	No

Site	Location <sup>†</sup>	GPS (site)	Date (m/d/y)	Number of <i>C. gloeosporioides</i>	<i>C. arceuthobii</i> observed (yes/no)
		122° 08' 49"W			
28	Quesnel	52° 32' 19"N	7/8/98	11	Yes
		122° 38' 25"W			
29	Quesnel	52° 31' 15"N	7/8/98	2	Yes
		122° 42' 13"W			
30	Lytton	50° 28' 20"N	7/9/98	3	No
		121° 37' 39"W			
31	Nordegg, AB	52° 00' 14"N	8/13/98	14	No
		116° 39' 45"W			
32 <sup>§</sup>	Bruderheim, AB	53° 52' 32"N	8/14/98	0	0
		112° 58' 36"W			
33	Tete Jaune	52° 58' 23"N	8/17/98	6	No
	Cache	119° 25' 54"W			
34	Valemount	52° 47' 34"N	8/18/98	6	Yes
		119° 15' 30"W			
35	Lac le Jeune	50° 18' 47"N	8/18/98	5	No
		120° 38' 30"W			
36	Canal Flats	50° 07' 44"N	8/12/98	12	No
		116° 00' 30"W			
Na	Grand Forks <sup>‡</sup>	Na	6/9/98	1	No

<sup>†</sup> Nearest town

<sup>§</sup> Collected from *A. americanum* parasitizing *Pinus banksiana*

<sup>‡</sup> Collected by Dr. Dick Smith, no GPS

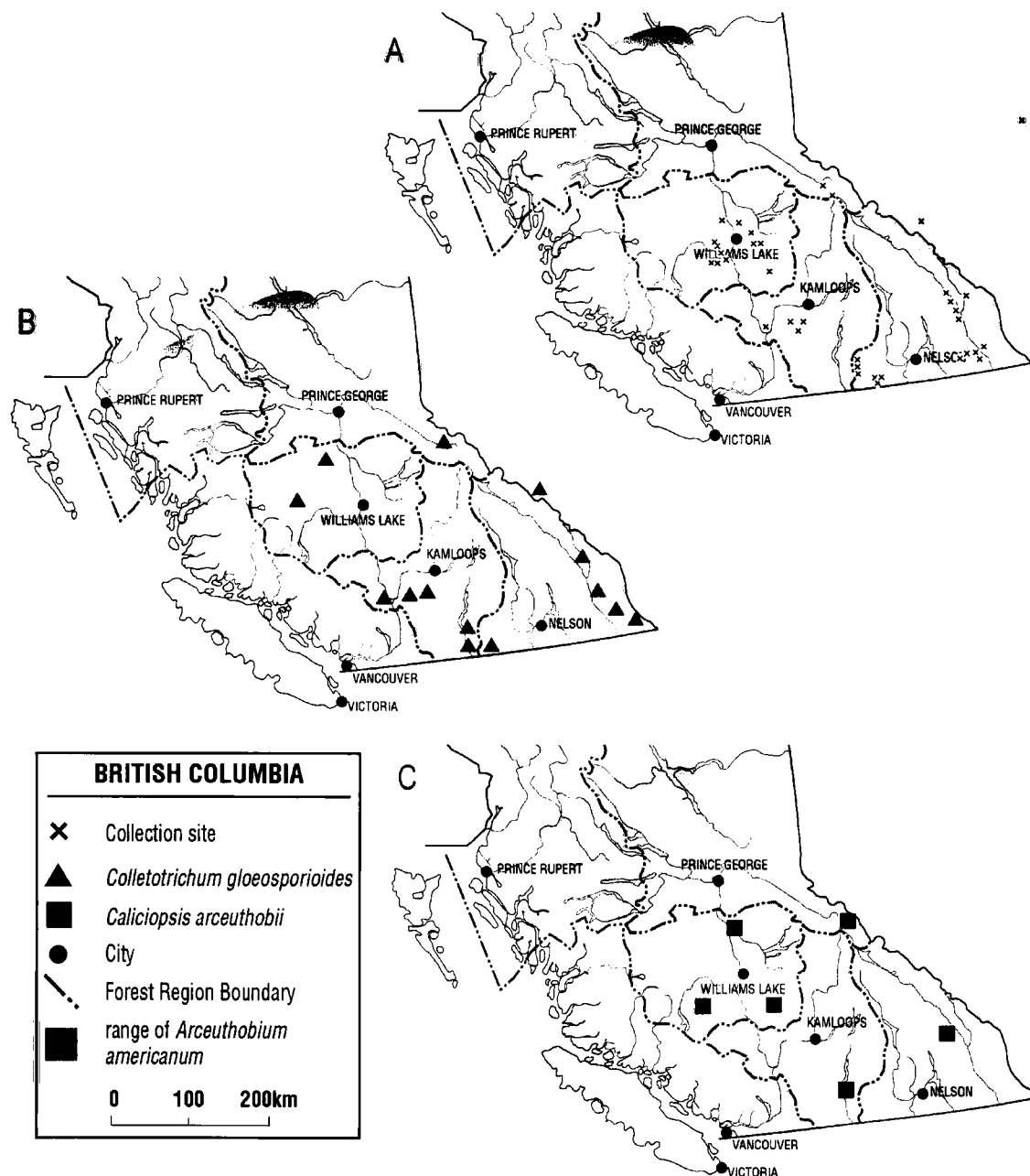
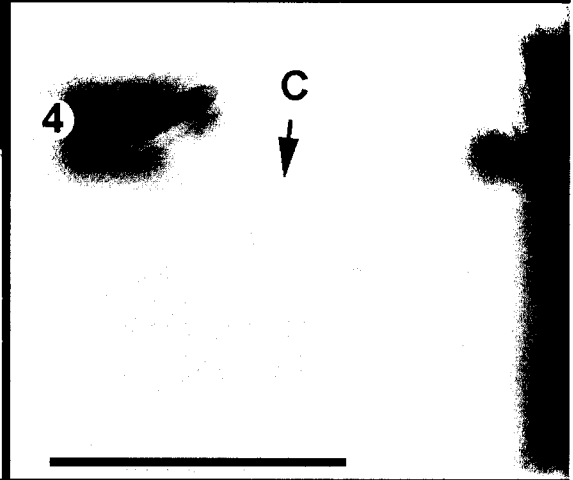
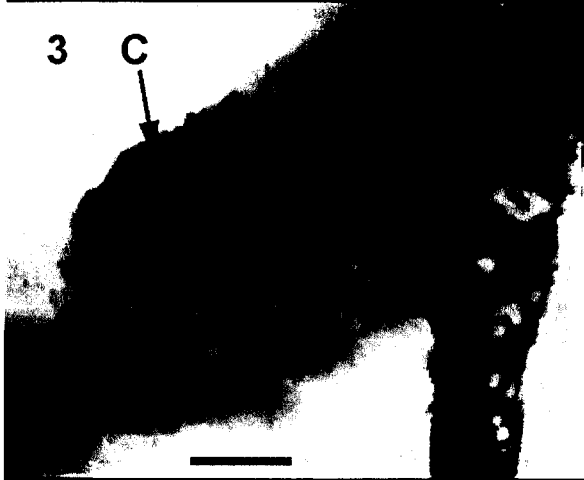
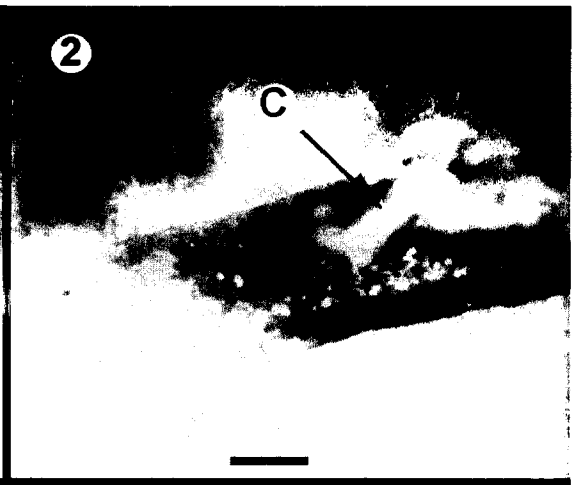
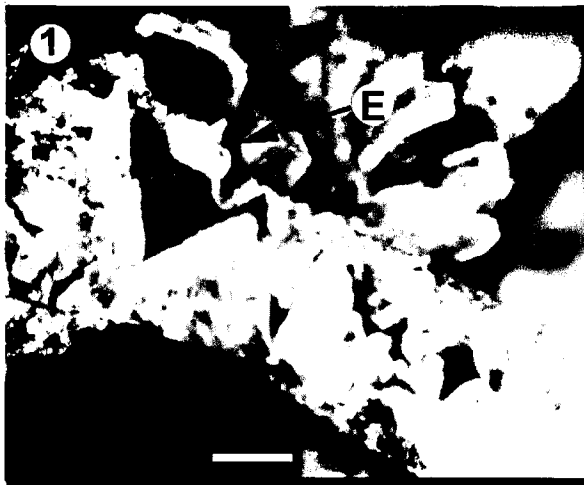


Figure 2-2. Geographic location of: A. *Arceuthobium americanum* collection sites, B. Sites where *Colletotrichum gloeosporioides* isolates were collected and, C. Where *Caliciopsis arceuthobii* was observed during this study.

*Colletotrichum gloeosporioides* (Melanconiales, Barnett and Hunter, 1998) (anamorphic stage of *Glomerella cingulata*) infection of *Arceuthobium americanum* resulted in brown to black necrotic regions at the shoot node, which eventually coalesced and ruptured through the shoot epidermal layer to expose black acervuli. Under moist conditions, masses of pink conidia were formed on the acervuli. Acervuli and conidia production were also observed on *A. americanum* fruit. The conidia were 12.5 – 20 x 2.5 – 5 µm, non-septate and straight. Typical melanized appressoria of 5 x 10 µm formed when germ tubes from germinated conidia contacted the bottom of the petri plate. In culture on malt extract agar (MEA), the fungal colonies had a grey center with dark brown transitioning to golden brown at the colony margin and scattered black conidiomata. On PDA, the young mycelium was fluffy, white, transitioning to mottled grey / green. Abundant conidia were produced on both MEA and PDA. The symptoms and signs of disease, as well as the conidia and appressoria characteristics agree with descriptions in Parmeter et al. (1959) and Sutton (1980) and are shown in figure 2-3. An *A. americanum* infection with typical *C. gloeosporioides* lesions, collected from Logan Lake, was deposited in the Forest Pathology Herbarium at the Pacific Forestry Centre (DAVFP 25861).

Figure 2-3. *Colletotrichum gloeosporioides* infection of *Arceuthobium americanum* and morphological characteristics. 1. Lesion on shoot, scale bar represents 1 mm, E = epidermis. 2. Acervuli exuding conidia (C), scale bar represents 1 mm. 3. Conidia (C) production on diseased fruit, scale bar represents 1 mm. 4. Conidia (C), scale bar represents 100  $\mu$ m. 5. Appressoria (A), scale bar represents 100  $\mu$ m. 6. Culture on MEA.



*Caliciopsis arceuthobii* was identified based on the presence of clusters of shiny, black perithecia that were present on the stylar region of the female flowers (Figure 2-4).

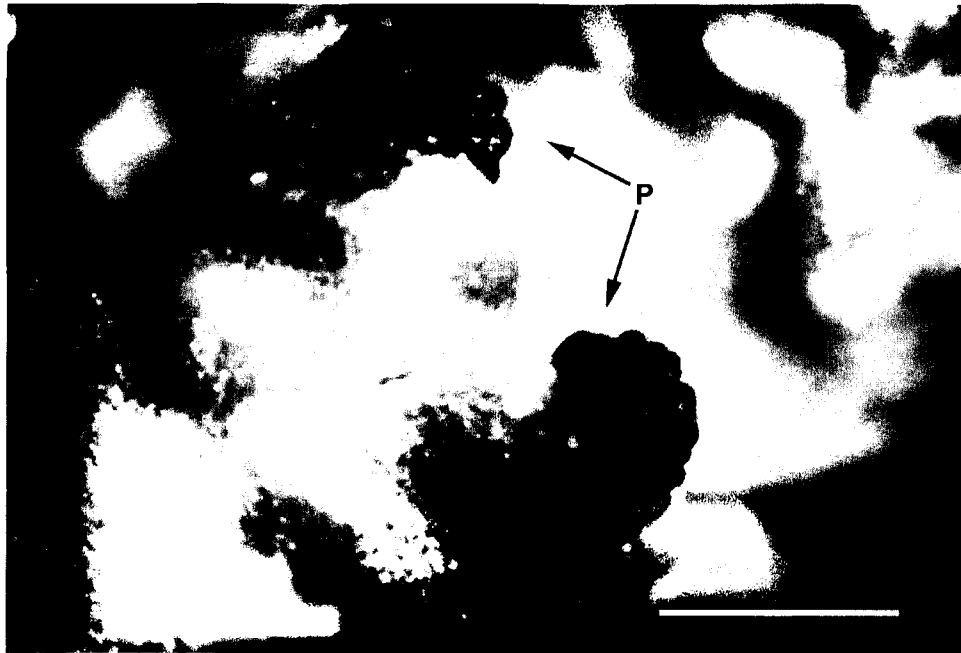


Figure 2-4. Perithecia (P) of *Caliciopsis arceuthobii* infecting female flowers of *Arceuthobium americanum*. Scale bar represents 1 mm.

### 2.3.2 Growth characteristics of *Colletotrichum gloeosporioides*.

The temperature profile of mycelial growth, spore germination and germ tube elongation of 4 isolates of *C. gloeosporioides* was measured at temperatures ranging from 0°C to 35°C. Figure 2-5 shows the average growth of each isolate at each temperature after 12 days incubation, before the growth rate began to slow (Figure 2-6). Maximum mycelial growth of PFC-4277, PFC-4493 and PFC-4501 occurred at 20°C, while maximum growth of PFC-4278 occurred at 25°C. Although isolate PFC-4278 did not grow as rapidly at 20°C as the other isolates, growth of this isolate at 15°C, 20°C, and 25°C was very uniform. All isolates grew well at temperatures ranging from 15°C to 25°C and growth was dramatically reduced at 10°C and 30°C.

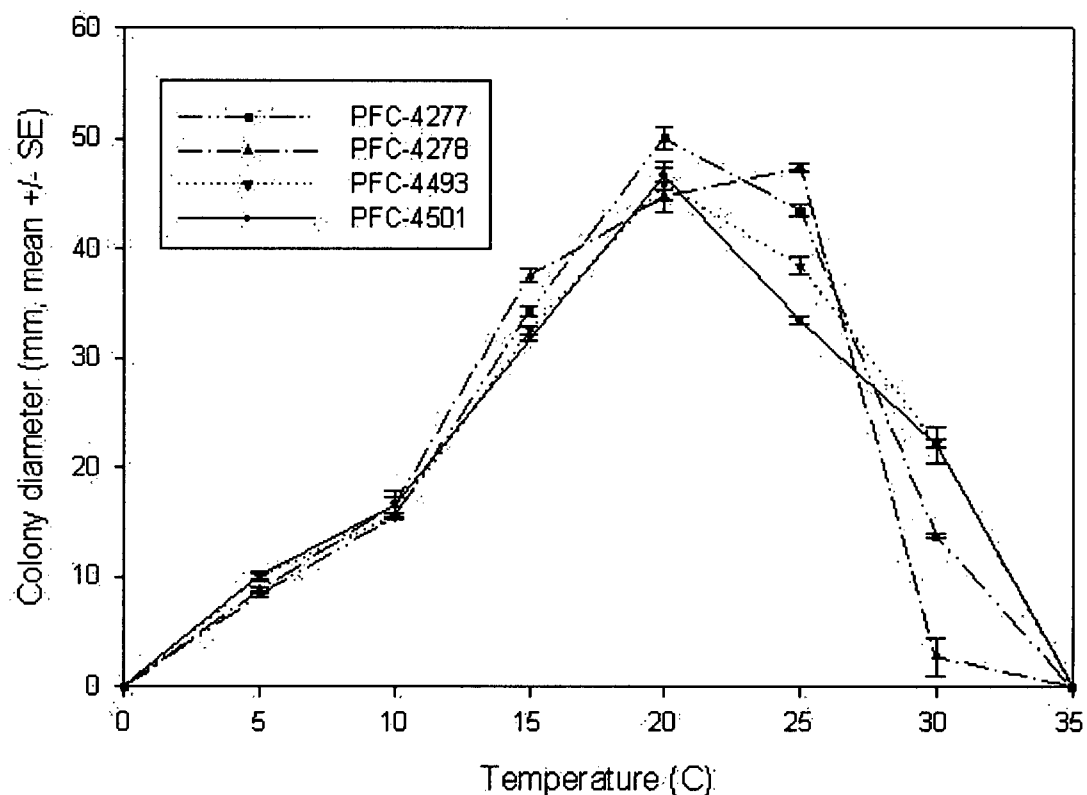


Figure 2-5. *C. gloeosporioides* colony diameter (mm) after 12 days incubation at temperatures ranging from 0°C to 35°C. Points plotted are the mean  $\pm$  standard error of the mean.

Plotting colony diameter growth over time at 20°C revealed that the increase in diameter was linear for the first 10 to 12 days, with a very slight decrease in rate over the remainder of the experiment, and that isolate PFC-4277 had the highest daily rate of growth (Figure 2-6).

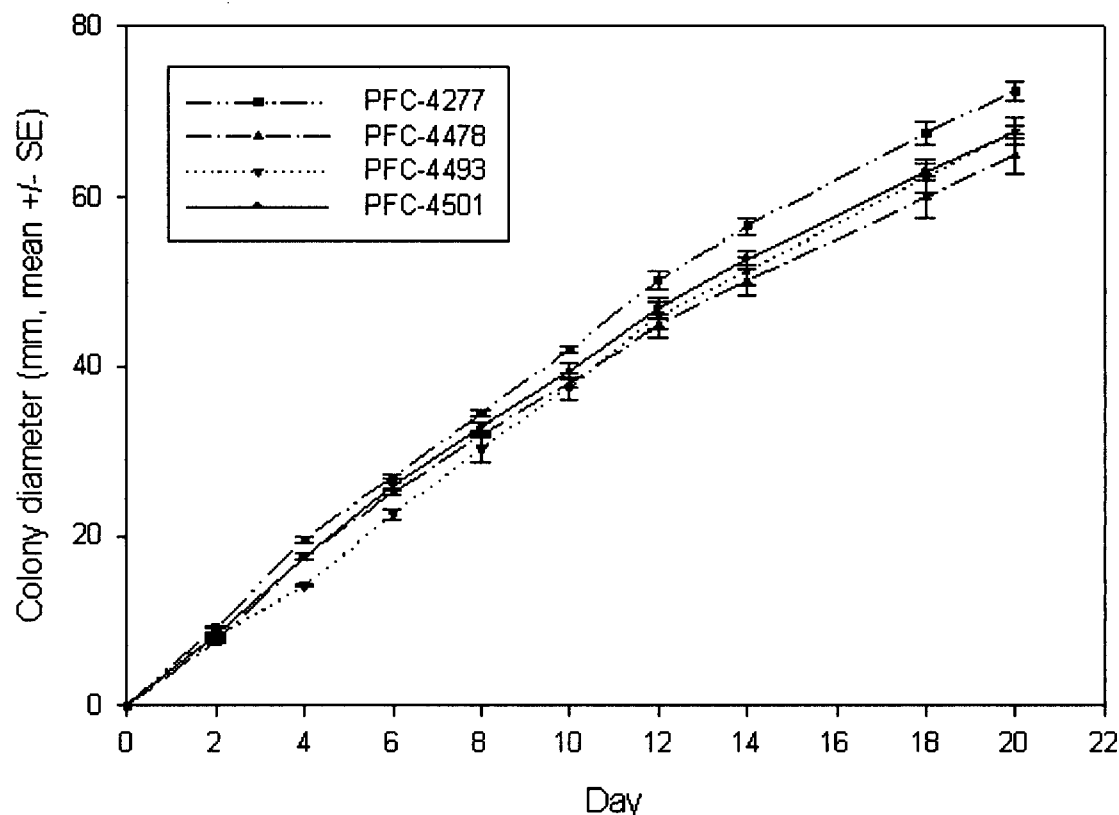


Figure 2-6. Colony growth. Increase in colony diameter of *C. gloeosporioides* incubated at 20°C over 20 days. Mean  $\pm$  standard error of the mean.

Conidia germination of the four isolates at temperatures ranging from 0°C to 35°C after 18 hours incubation was highly variable between isolates (Figure 2-7). Isolate PFC-4277 had at least 95% conidia germination at temperatures ranging from 10°C to 30°C, with a maximum rate of 97% at 25°C, indicating that the conidia were able to germinate over a very broad range of temperatures. Conidia of isolate PFC-4501 also germinated over a broad range of temperatures but maximum percent germination was only 70% at 15°C. Isolates PFC-4278 and PFC-4493 had poor conidial germination, with maximum germination of 54% and 42% respectively at 30°C. The selected isolates had maximum spore germination rates at temperatures that ranged from 15°C to 30°C.

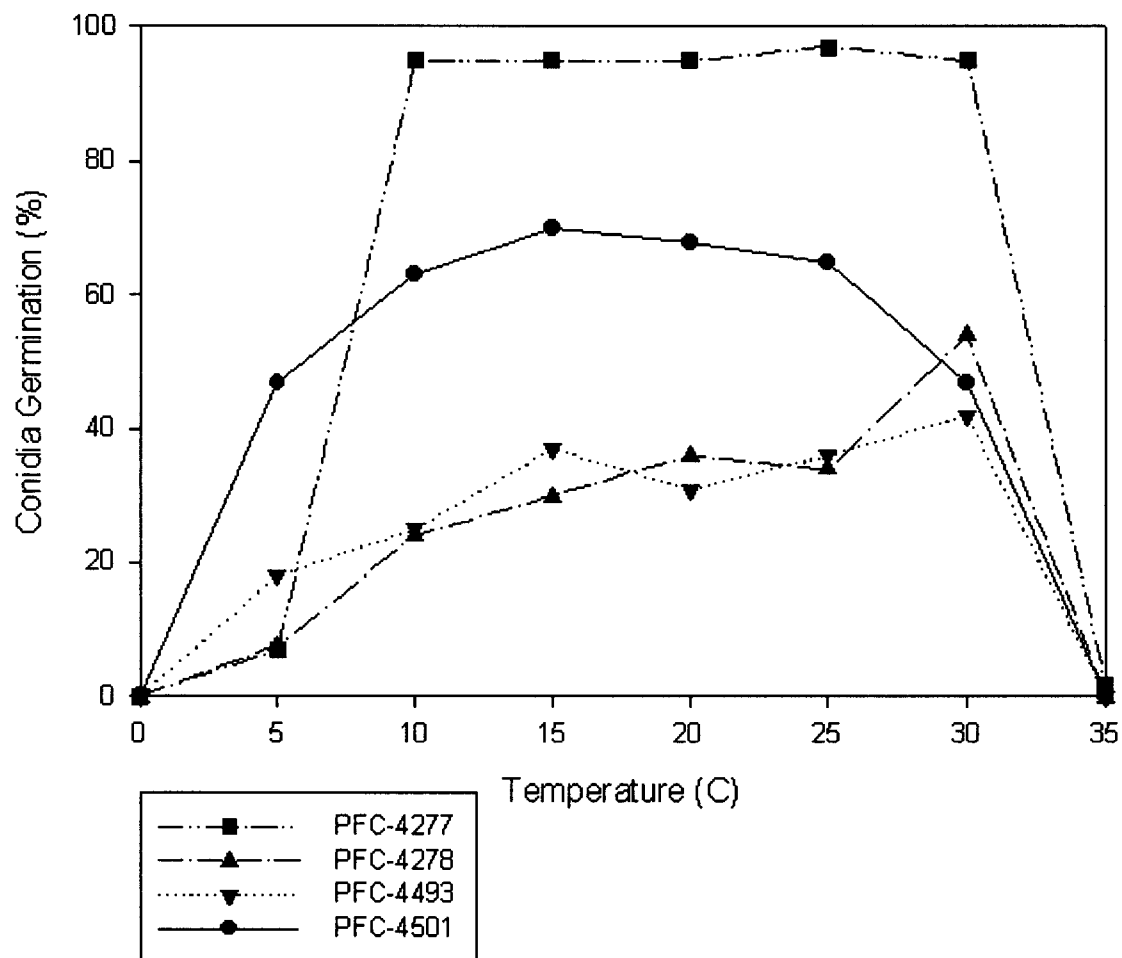


Figure 2-7. Percent conidial germination of *C. gloeosporioides* isolates at temperatures ranging from 0°C to 35°C.

Germ tube length at temperatures ranging from 0°C to 35°C after 18 hours incubation was maximum at 25°C for all isolates and PFC-4277 had the maximum germ tube growth, with maximum germ tube length averaging 265  $\mu\text{m}$ . As with colony diameter growth, isolate PFC-4278 did not have the maximum germ tube length, but this isolate had the least amount of change between 20°C and 25°C (Figure 2-8).

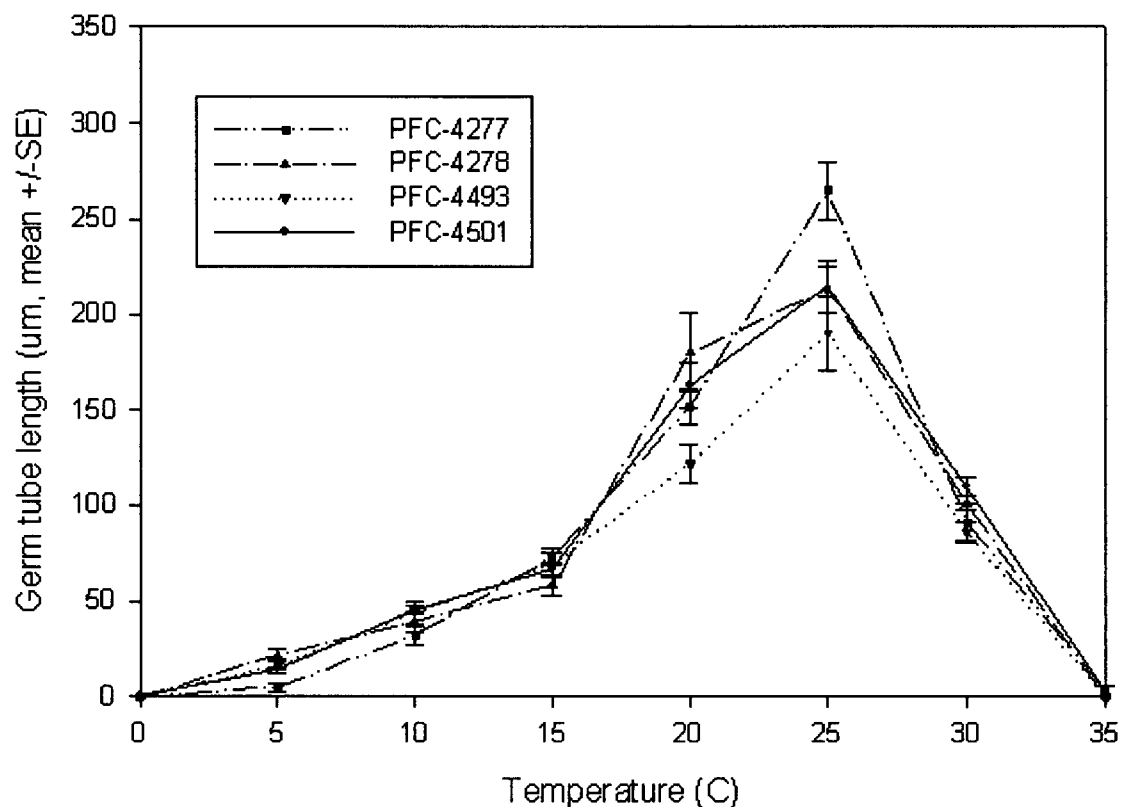


Figure 2-8. Germ tube elongation at temperatures ranging from 0°C to 35°C after 18 hours incubation. Mean +/- standard error of the mean.

In all measured characteristics, the growth of *C. gloeosporioides* was inhibited at 0°C and 35°C and growth was very slow at 5°C and 30°C. Percent conidia germination was highly variable between isolates, while the growth characteristics were much more consistent.

The growth characteristics of the four isolates were compared and utilized to determine which isolate should be selected as the lead biological control isolate. Isolate PFC-4277 had the maximum diameter growth at 20°C, the maximum hyphal growth rate at 20°C, at least 95% conidia germination at temperatures ranging from 5°C to 30°C and the maximum rate of germ tube elongation. Additionally, conidia production by PFC-4277 was an order of magnitude higher than the other isolates. After conidia were harvested from the millet grain, PFC-4277 was found to produce  $2.035 \times 10^8$  conidia per

ml, while PFC-4493 had the second highest conidia concentration at  $1.725 \times 10^7$  conidia per ml and PFC-4501 and PFC-4278 produced  $1.55 \times 10^7$  and  $1.1 \times 10^7$  conidia per ml respectively.

### **2.3.3 Formulation**

Sodium alginate – kaolin clay formulation resulted in the formation of very uniform pellets of approximately 3 mm in diameter after drying. Following 108 days storage, *C. gloeosporioides* had emerged from 16/16 replicates of the 4°C stored formulation after 48 hours, while at 72 hours *C. gloeosporioides* had emerged from only 4/16 replicates of the lot stored at room temperature. When the 4°C stored formulation was ground to a powder using a mortar and pestle and placed on PDA, the fungus emerged from the powder within 24 hours. Powdered formulation was suspended in a corn oil emulsion and plated onto PDA. After 5 days incubation, all emulsions up to 25% oil had 100% fungal growth, while the 50% oil emulsion had growth from 7/12 spots, 75% oil emulsion had 1/12 spots and 100% oil had no growth. The separation of the oil and water components of the emulsion occurred rapidly. The ground powder did not distribute well within the formulation, clogged the pipette tip easily and it settled out of solution very quickly. Due to the difficulty encountered suspending the powder, the rapid separation of the oil and water emulsion, as well as the negative effect of the oil on the fungus, the approach was abandoned. In the small scale greenhouse trial on *A. tsugense*, the ground formulation was sprinkled onto *A. tsugense* swellings and shoots that were soaked with a sucrose-gelatin solution and then hydrated by spraying more of the sucrose-gelatin solution on the treated areas.

### **2.3.4 Inoculation of *Arceuthobium tsugense* with sodium alginate formulated *Colletotrichum gloeosporioides* under greenhouse conditions.**

Two isolates of *C. gloeosporioides* were used: PFC-4277 isolated from *A. americanum* and PFC-2329 isolated from *A. tsugense* (Table 2-1). The fungus was applied after formulation in sodium alginate – kaolin clay, as well as a conidial suspension (PFC-4277 only). The control treatments, 2% sucrose and 0.5% gelatin solution and 2% sucrose and 0.5% gelatin solution plus sterile sodium alginate – kaolin

clay formulation, had no effect on the dwarf mistletoe. Thirteen days following inoculation, one replicate treated with the conidial suspension of PFC-4277 had two dead and two healthy shoots. One of the dead shoots from this replicate was sampled and *C. gloeosporioides* was isolated from the necrotic tissue. This was the only replicate of any treatment that became diseased as a result of *C. gloeosporioides* application. Application of the isolates of *C. gloeosporioides* formulated in sodium alginate – kaolin clay had no effect on *A. tsugense*. The powder remained on the dwarf mistletoe shoots for the duration of the experiment, but it is likely that the fungus never emerged from the formulation. The cut shoot treatment resulted in immediate necrosis at the cut ends of the shoots, but the tree showed symptoms of root damage 13 days after inoculation and was removed from the experiment, before the results of *C. gloeosporioides* infection on the emergence of new shoots could be assessed. The experiment was monitored for 55 days and was ended at this point because an outbreak of black vine weevils in the greenhouse compartment resulted in damage to 14 of the 15 remaining dwarf mistletoe plants; the aerial systems of 8 of the infections were completely destroyed.

## **2.4 Discussion**

### **2.4.1 Collection of diseased *A. americanum* and hyperparasite isolation**

The first step in the development of a biological control agent is the selection of a pathogen that causes disease on the target organism. Therefore, the first objective of this research was to survey the range of lodgepole pine within British Columbia and to isolate fungi that caused disease on *A. americanum*. The presence of *Caliciopsis arceuthobii*, *Cylindrocarpon gillii* and *Colletotrichum gloeosporioides* infecting *Arceuthobium* spp. in British Columbia have been reported (Wood, 1986) and these hyperparasites were sought in this study. *Colletotrichum gloeosporioides* was isolated from *A. americanum* at 24 of 36 sites visited (Table 2-2) and was distributed throughout the southern range of *A. americanum* in British Columbia (Figure 2-2, panel B). *Caliciopsis arceuthobii* was observed at 8 sites (Figure 2-2, panel C), however the fungus was not isolated in pure culture. The collection time or the slow rate of growth of *C. arceuthobii* were the likely factors that prevented isolation in pure culture. Parker (1970) isolated *C. arceuthobii*

from perithecia that were collected in October, November and April, and Knutson and Hutchins (1979) isolated it from December collections, while in this study, *C. arceuthobii* was collected in May, June, July and August, possibly after ascospores were released from the perithecia and before perithecia were present on newly infected female flowers. Perithecia were plated directly onto the glucose-asparagine media suggested by Parker (1970), however mycelial growth of *C. arceuthobii* was not recovered, possibly because *C. arceuthobii* grows very slowly on media (Knutson and Hutchins, 1979; Parker, 1970) and the fungus was overgrown by a *Cladosporium* sp. present on the perithecia. *Cladosporium* sp. has been reported to grow on the perithecia of *C. arceuthobii* after ascospore liberation from the perithecia (Kjuit, 1969) and it was isolated from perithecia in this study. *Cylindrocarpon gillii* was not recovered or observed during the survey. *Cylindrocarpon gillii* was first observed on *A. americanum* in Montana by Gill (1952) and three accessions of this fungus from British Columbia are stored in the forest pathology herbarium at the Pacific Forestry Centre in Victoria (DAVFP 15077, DAVFP 15081, DAVFP 24008). The herbarium samples were examined, but they were old and discoloured and it was difficult to differentiate them from herbarium samples of *C. gloeosporioides*. Microscopic examination of spores from the herbarium samples was not conducted. The range of *C. gillii* in British Columbia is much more restricted than *C. gloeosporioides* and *C. arceuthobii* (Wood, 1986), therefore, it is possible that had more sites been visited, *C. gillii* would have been observed on *A. americanum* in this study.

It was decided to focus on *C. gloeosporioides* as a potential inundative biological control agent for *A. americanum* because it meets the requirements outlined by Wicker and Shaw (1968) and *Colletotrichum* spp. have been utilized as biological control agents in other pathosystems (Templeton, 1992). The range of *C. gloeosporioides* was found to coincide with the range of *A. americanum* that was surveyed in British Columbia and was readily isolated from diseased *A. americanum*. As the fungus is native to *A. americanum* in British Columbia, the risks associated with importing exotic pathogens as biological control agents are eliminated. Cultural characteristics that make *C. gloeosporioides* amenable to development as a biological control agent include rapid growth and abundant inoculum production in culture. The fungus was observed to infect and kill male and female shoots in all stages of development, as well as infect fruit, and it has been reported

to infect the endophytic system (Parmeter et al., 1959). Artificial inoculation by Parmeter et al. (1959) resulted in symptoms of disease 13 days after inoculation and girdling lesions 21 days after inoculation. In contrast, *C. arceuthobii* only infects female dwarf mistletoe flowers, grows very poorly in culture and the life cycle requires two seasons for completion (Knutson and Hutchins, 1979).

#### **2.4.2 Growth characteristics of *Colletotrichum gloeosporioides*.**

The growth characteristics of 4 isolates of *C. gloeosporioides* were investigated for the purpose of lead isolate selection for future biological control work. The isolates originated from three different geographic locations, representing different areas of British Columbia. Conidial production in culture was quantified, colony diameter growth rate was measured, conidial germination and germ tube elongation at temperatures ranging from 0°C to 35°C. Isolate PFC-4277 was selected as the lead isolate because it had the highest conidia production in culture, the fastest mycelial growth and highest conidia germination rates. Although isolate PFC-4278 had little variation in mycelial growth and germ tube elongation at temperatures ranging from 15°C to 25°C and 20°C and 25°C, respectively, this isolate was not selected as the lead isolate because the percent conidia germination was low. The percent conidia germination of different isolates at different temperatures described in figure 2-8 was highly variable.

When germination was quantified, all plates were removed from treatment conditions at the same time and placed in a refrigerator at 4°C, which had the effect of greatly reducing the rate of growth, but the last plates to be assessed were in the refrigerator for approximately 5 hours. Percent conidia germination over time was not measured, and it is probable that after a longer incubation time, isolates PFC-4278 and PFC-4493 would have approached the level of isolate PFC-4277. Conidia germination decreases with increasing conidia concentration (Morin et al., 1996), possibly as a result of inhibitory compounds in the conidial matrix (Mondal and Parberry, 1992). The concentration of conidia that was utilized in this experiment was  $1 \times 10^5$ , which was the concentration at which Morin et al. (1996) achieved maximum germination, therefore, the variability of germination observed between isolates is not likely a result of self inhibition due to over crowding. The variability of conidial germination observed in this

study was corroborated in a study of *C. gloeosporioides* isolates that were collected from mango leaves in the Philippines (Estrada et al. 2000). The authors recorded 100% conidia germination at 20°C, 25°C and 30°C for one isolate after 15 hours, while another isolate reached 100% germination at the three temperatures after 30 hours. In both isolates, spore germination occurred most rapidly at 25°C and 30°C (Estrada et al., 2000). Speed of conidial germination is one of the most important characteristics of a biological control agent. As the length of time between application and conidial germination increases, the probability of successful establishment decreases because abiotic factors such as relative humidity and ultraviolet light damage will decrease the viability of the conidia. The formulation process is designed to reduce the effects of abiotic factors to increase the probability of establishment; however, rapid conidia germination is also an important component of the infection process.

The growth characteristics measured provided an indication of which isolate may be the best isolate for field inoculation based on assumptions relating to the relationship between conidia production, germination and fungal establishment. Virulence was not assessed directly because no experimental units of *A. americanum* were available for testing under controlled conditions. The isolate selected for formulation may not be the most virulent of the selected isolates, but without the ability to directly assess virulence on *A. americanum*, it is impossible to determine if the most virulent isolate was selected.

#### **2.4.3 Formulation**

Formulation of the biological control agent is a process whereby the active ingredient is mixed with inert carriers to enhance long term storage without loss of viability and biological activity (Boyette et al., 1996). It is an important step in the development of a biological control strategy because the formulation provides moisture to reduce or eliminate the dew period requirement (Boyett, 1994), allows application with conventional spray systems (Egley and Boyette, 1995), gives protection against UV-irradiation and provides nutrition to the fungal propagules (Neumann and Boland, 1999), thus enhancing the efficacy of the biological control agent

Formulation of *C. gloeosporioides* using the sodium alginate – kaolin clay method resulted in the formation of pellets that were easy to handle and provided stable long term

storage at 4°C. Grinding the pellets with a mortar and pestle likely caused a reduction in inoculum viability as a result of cell wall shearing and heat generation, but the reduction was not quantified. The fungus did emerge from ground pellets, indicating that some of the formulated conidia remained viable. Inhibition by the corn oil emulsion was found to increase with increasing corn oil concentration and it was difficult to work with; therefore, the formulation application method was modified for the greenhouse trial. A solution composed of 2% sucrose and 0.5% gelatin (Trujillo et al., 1994) was used to hydrate the formulation, provide a readily available carbon source to the fungus and to allow it to adhere it to the target dwarf mistletoe.

#### **2.4.4 Inoculation of *Arceuthobium tsugense* with *Colletotrichum gloeosporioides* under greenhouse conditions.**

A small scale trial under greenhouse conditions was initiated to test the ability of sodium-alginate kaolin clay formulated *C. gloeosporioides* to infect *A. tsugense* infecting *T. heterophylla*. *Arceuthobium tsugense* was not the primary target organism of this research, however, experimental units of *A. americanum* that could be inoculated under greenhouse conditions were not available. The greenhouse study was therefore established with the primary intention of testing an inoculation strategy prior to use with *A. americanum* under field conditions and to verify Koch's postulates. An isolate of *C. gloeosporioides* that was collected from *A. tsugense* was included in the trial to avoid any host / parasite specializations that may exist. To test the susceptibility of *A. tsugense* to *C. gloeosporioides* collected from *A. americanum* an isolate that was collected from *A. americanum* was also included in the study.

Infection of *A. tsugense* from the formulated *C. gloeosporioides* did not occur in the highly controlled environment of the greenhouse following 60 hours of near 100% humidity, implying that it is unlikely that the fungus would become established if applied in this manner under field conditions. Grinding the formulation did not result in 100% mortality of the fungus; therefore, it is likely that the fungus did not become established from the formulation because the moisture available to the formulated conidia was too low. A conidial suspension of *C. gloeosporioides* isolate PFC-4277, collected from *A. americanum*, did however become established on, and was re-isolated from, *A. tsugense*

suggesting that the fungus was able to cause disease on a range of species of *Arceuthobium* and that the environmental conditions available to the fungus following inoculation were satisfactory for establishment. The results of this experiment were also important because it was proven that it is possible for *C. gloeosporioides* isolated from *A. americanum* cause disease on *A. tsugense*, confirming the results of Scharpf (1964) and the lack of host specificity.

The effect of the black vine weevils, as well as the failure of sodium alginate – kaolin clay formulated *C. gloeosporioides* to infect *A. tsugense*, prevents assessment of *C. gloeosporioides* as a biological control agent for *A. tsugense*. Although the effect of the fungus could not be assessed, the results of the greenhouse trial were valuable because they pointed out the weakness of the formulation. This resulted in the initiation of the search for an alternative formulation method for the field trial on *A. americanum*.

## Chapter 3 – Development of a biological control strategy for *Arceuthobium americanum*, Part II: Field trial

### 3.1 Introduction

It was decided to focus on *Colletotrichum gloeosporioides* as an inundative biological control agent for *Arceuthobium americanum*. Prior to inoculation in the field, it is preferable to perform inoculation studies under controlled conditions. The small greenhouse trial described in Chapter 2 allowed testing of an inoculation technique but did not provide efficacy data. To generate experimental units of *A. americanum* for such inoculation, *A. americanum* seeds were collected and placed on lodgepole pine seedlings in the greenhouse. It is known that a minimum incubation time of 2 years is required before aerial shoots are produced (Hawksworth and Wiens, 1996), and unfortunately, no experimental units were ready in time for this study. Due to the lack of experimental units for greenhouse testing, the study proceeded to field inoculation of *A. americanum* with a *C. gloeosporioides* isolate that was selected based on growth characteristics rather than a direct assessment of virulence.

The infection process of *C. gloeosporioides* is initiated when conidia, produced on acervuli, are released during rain and dew (Wastie, 1972) and disease severity increases with increasing rain (Carrington et al., 2001). Conidia are encapsulated by a conidial matrix composed of exopolysaccharides and glycoproteins (Mondal and Parbery, 1992), that serves to prevent conidial germination before dispersal and to maintain germinability during periods of environmental stress (Louis and Cooke, 1985). Following splash dispersal (Yang et al., 1990), conidia must adhere to the plant surface. The exact mechanism of conidial adhesion is unknown, but it is not likely that specific adhesives are released from the conidia (Bailey et al., 1992). Pathogenesis is initiated by the formation of melanized appressoria, which penetrate the host epidermis without restriction to a specific host structure (Makowski and Mortensen, 1998). The dew period required for successful establishment on the host is directly correlated with the timing of germination, appressoria formation and penetration (Makowski and Mortensen, 1998; Makowski, 1993). *Colletotrichum gloeosporioides* has a two-stage pathogenesis process, termed intracellular hemibiotrophy. The initial phase of infection is symptomless and

occurs after penetration of the epidermal layer. Infection vesicles are formed in infected epidermal cells, but the cytoplasm of the epidermal cell is invaginated around the vesicle. Large diameter primary hyphae emerge from the infection vesicle and enter adjacent epidermal and cortical cells. The primary hyphae are constricted as they pass through the cell and the infected cell retains its ultrastructure and membrane function for 24 to 48 hours following penetration. After 2 to 3 days, the cytoplasm of the cell has degenerated and only membrane debris are present. The primary hyphae proceed from cell to cell and there is a slow transition to cell death (Bailey et al., 1992). On some hosts, *C. gloeosporioides* forms latent infections, whereby conidia germinate, form appressoria and penetrate the epidermal layer and form infection hyphae that remain latent within the upper cell layers. When the host plant becomes stressed through ripening, wounding or senescence, the membrane permeability of the host cells is changed and soluble nutrients are released that may trigger the end of latency (Cerkauskas, 1988). Following the initial biotrophic phase, thin secondary hyphae are formed that cause degradation and death of cells by intracellular and intramural growth during the necrotrophic phase of the pathogen. The necrotrophic phase is responsible for the formation of anthracnose symptoms and the fungus is able to grow throughout host tissues causing extensive cellular destruction through the production of polygalacturonases and pectin lyase that degrade cell walls. *Colletotrichum gloeosporioides* also produces low molecular weight phytotoxins, however, the exact role of these phytotoxins in the pathogenesis process is unknown (Bailey et al., 1992).

*Colletotrichum gloeosporioides* is widespread on many species of *Arceuthobium* in the western United States and Canada (Hawksworth et al., 1977). Initially described on *Arcethobium abietinum* by Parmeter et al., (1959), the fungus has been suggested as a biological control agent for dwarf mistletoes (Parmeter et al., 1959, Wicker, 1967; Knutson, 1978), however, the only study in which dwarf mistletoe was inoculated with *C. gloeosporioides* was by Parmeter et al., (1959). Other studies have surveyed infected dwarf mistletoe stands and estimated the natural control rate of *C. gloeosporioides*. Wicker (1967) indicated that *C. gloeosporioides* provided a significant level of natural control of *A. campylopodum*, while Muir (1977) found that *C. gloeosporioides* did not provide sufficient control of *A. americanum*. Although *C. gloeosporioides* can be

destructive, the overall effect of the fungus was judged to be minor (Hawksworth, 1972), likely due to the fact that the fungus is a native pathogen on a native host (Hawksworth et al., 1977). The conclusion that *C. gloeosporioides* would not provide adequate biological control of dwarf mistletoe was drawn prior to the recent advent of formulation technology and inundative biological control. The objective of this study was to assess the potential of *C. gloeosporioides* as an inundative biological control agent for *A. americanum* under field conditions by observing symptom development and quantifying the reduction in fruit production following fungal application.

### **3.2 Materials and Methods**

#### **3.2.1 'Stabilize' formulation**

The sodium alginate – kaolin clay formulation was found to be unsatisfactory for inoculation of dwarf mistletoe, therefore, the 'Stabileze' method developed by Quimby et al. (1999) was utilized to encapsulate *C. gloeosporioides*. Prior to formulation, *C. gloeosporioides* conidia of isolate PFC-4277 were harvested from millet grain by washing with sterile distilled water using the same technique as was employed to collect conidia for the germination study (Chapter 2). The harvested conidia were quantified using the hemacytometer and the volume of the conidial suspension was adjusted with sterile distilled water to  $1.0 \times 10^7$  conidia per ml. The conidia were then formulated as follows: 16.656 g Waterlock® B-204 (Grain Processing Corp., Muscatine, IA) was mixed with 16.7 ml of corn oil and then heated on 'high' in a microwave oven for 2 minutes. Once the mix had cooled to room temperature, 66.625 ml of conidial suspension was added and 66.625 g sucrose and 23.32 g Hi-Sil® 233 (PPG Industries Inc., Pittsburgh, PA) were slowly added and mixed into the conidia / Waterlock® B-204 / corn oil solution. The resulting powder was spread onto foil covered trays and allowed to air dry. A sterile control formulation was also made by substituting sterile distilled water for the conidial suspension. The dried formulation was weighed and stored at 4°C until required. The formulation was prepared for inoculation by placing 5 g of the dried formulation into 500 ml of sterile distilled water, resulting in a final concentration of approximately 53,000 conidia per ml, stirred for 30 minutes and then placed in a hand

atomizer. To inoculate *A. americanum* the formulation was sprayed onto the aerial shoots and swelling to run off.

### 3.2.2 Site description

The field site was located in the Lillooet District of the Kamloops Forest Region. The site was approximately 2 hectares in size and was classified as the dry cool variant of the Interior Douglas Fir zone (IDF dk1) following the biogeoclimatic ecosystem classification system employed by the British Columbia Ministry of Forests (Lloyd et al., 1990). The overstory was composed of *A. americanum* infected lodgepole pine of 80 - 100 years and the understory was composed of *A. americanum* infected lodgepole pine of approximately 30 years. All of the *A. americanum* infections included in the experiment were located in the lower crown of the understory trees. A Hobo data logger (Onset, Bourne, MA) was installed near the center of the plot to record temperature and relative humidity (%) at 15 minute intervals from 1 week prior to inoculation day until 6 weeks following inoculation.

### 3.2.3 Experimental design

*Arceuthobium americanum* infections were selected for inclusion in the field trial if they met the following criteria: *A. americanum* shoots were disease free, with well defined localized swellings (ie. no systemic infections) and a single *A. americanum* infection on the host branch. Male and female *A. americanum* infections were selected so that *C. gloeosporioides* symptom development could be monitored on as large a sample population as could be located within the inoculation site. Dwarf mistletoe age cannot be determined without destructive sampling, therefore, it is likely that a range of *A. americanum* ages was represented in the sample population.

A total of 255 individual *A. americanum* infections and 7 treatments were included in the field trial (Table 3-1). There were 45 replicates of treatments 1, 2, 3, 4 and 5 and 5 replicates of treatments 6 and 7. Treatments 6 and 7 were included as observational controls and were not included in the statistical analysis. The number of male and female dwarf mistletoe infections was equalized between treatments, with treatments 1, 2 and 3 having 16 male and 29 female infections per treatment, treatment 4

having 14 male and 31 female infections and treatment 5 having 15 male and 30 female infections. The number of female infections per treatment was equalized so that the effect of *C. gloeosporioides* on fruit production could be assessed. Multiple *A. americanum* infections on the same host tree received the same treatment to avoid translocation effects and treatments 1, 2 and 3 were each applied to *A. americanum* on 31 trees, treatment 4 was applied to 30 trees and treatment 5 was applied to 29 trees. The effect of relative humidity on the establishment of *C. gloeosporioides* on *A. americanum* was tested by randomly enclosing 22 replicates from each treatment within a clear polyethylene bag for 48 hours following inoculation. After application, PDA plates were sprayed with treatment 1 and the control 'Stabileze' used in treatment 4 to verify viability of *C. gloeosporioides* and sterility, respectively.

Table 3-1. Treatment description and number of replicates per treatment in the field trial designed to test the efficacy of *C. gloeosporioides* as a biological control agent for *A. americanum*.

Treatment #	Treatment description	# of replicates
1	Isolate PFC-4277 formulated in 'Stabileze' sprayed on <i>A. americanum</i> shoots to run off (two applications).	45
2	Isolate PFC-4277 formulated in 'Stabileze' applied directly to cut ends of <i>A. americanum</i> shoots cut 5 mm from the bark.	45
3	Isolate PFC-4277 applied as a mycelial plug to artificially wounded lodgepole pine bark in the center of the <i>A. americanum</i> swelling.	45
4	Formulation control: 'Stabileze' formulated with sterile distilled water in place of fungal conidia and sprayed to run off.	45
5	Check: The <i>A. americanum</i> infection was not treated.	45
6	Cut shoot control: <i>A. americanum</i> shoots were cut and not treated.	5
7	Mycelial plug control: sterile media was applied to artificially wounded lodgepole pine bark in the center of the <i>A. americanum</i> swelling.	5

Treatments 3 and 7 were applied as follows: 1. The bark was thoroughly wiped with a 95% ethanol soaked cheesecloth to reduce surface contamination, 2. The cutting

end of a #4 cork borer was dipped in 95% ethanol and a hole was cut in the bark of the lodgepole pine branch and the bark removed to expose the cambium, 3. A plug of *C. gloeosporioides* mycelium and PDA (treatment 3, mycelium side down) or sterile PDA (treatment 7) was placed in the wound, 4. The wound was wrapped with Parafilm M (American National Can, Chicago, IL) to prevent colonization of the PDA by airborne contaminants.

Treatments 2 and 3 were designed to inoculate the endophytic system of *A. americanum*. Dwarf mistletoe shoots are directly connected to the endophytic system and by inoculating the ends of cut shoots with *C. gloeosporioides*, a conduit was provided for the fungus to enter the endophytic system. Inoculation of the bark with *C. gloeosporioides* during treatment 3 application placed the fungus in close proximity to the endophytic system, possibly allowing fungal colonization. These treatments were included in the experimental design to observe the effect of *C. gloeosporioides* on new shoot production as well as to provide samples for destructive sampling that are required in Chapter 4.

### 3.2.4 Pre-treatment assessment

Prior to inoculation, the following data was recorded for every *A. americanum* infection: swelling diameter, swelling length, distance between extreme shoots, number of shoots, number of buds (shoots less than 5 mm in height), number of fruit, maximum shoot length, sex, and vigour rating (Table 3-2, Figure 3-1).

Table 3-2. Vigour rating classes developed to classify individual *A. americanum* infections.

Vigour rating	Description
0	Dead
1	Small infection with 1 or 2 shoots and few fruit (if female).
2	Small infection with few shoots and modest fruit (if female).
3	Average infection with approximately 10 shoots and fruit (if female).
4	Large infection with many shoots and fruit (if female)
5	Huge infection with many long shoots and large numbers of fruit (if female).

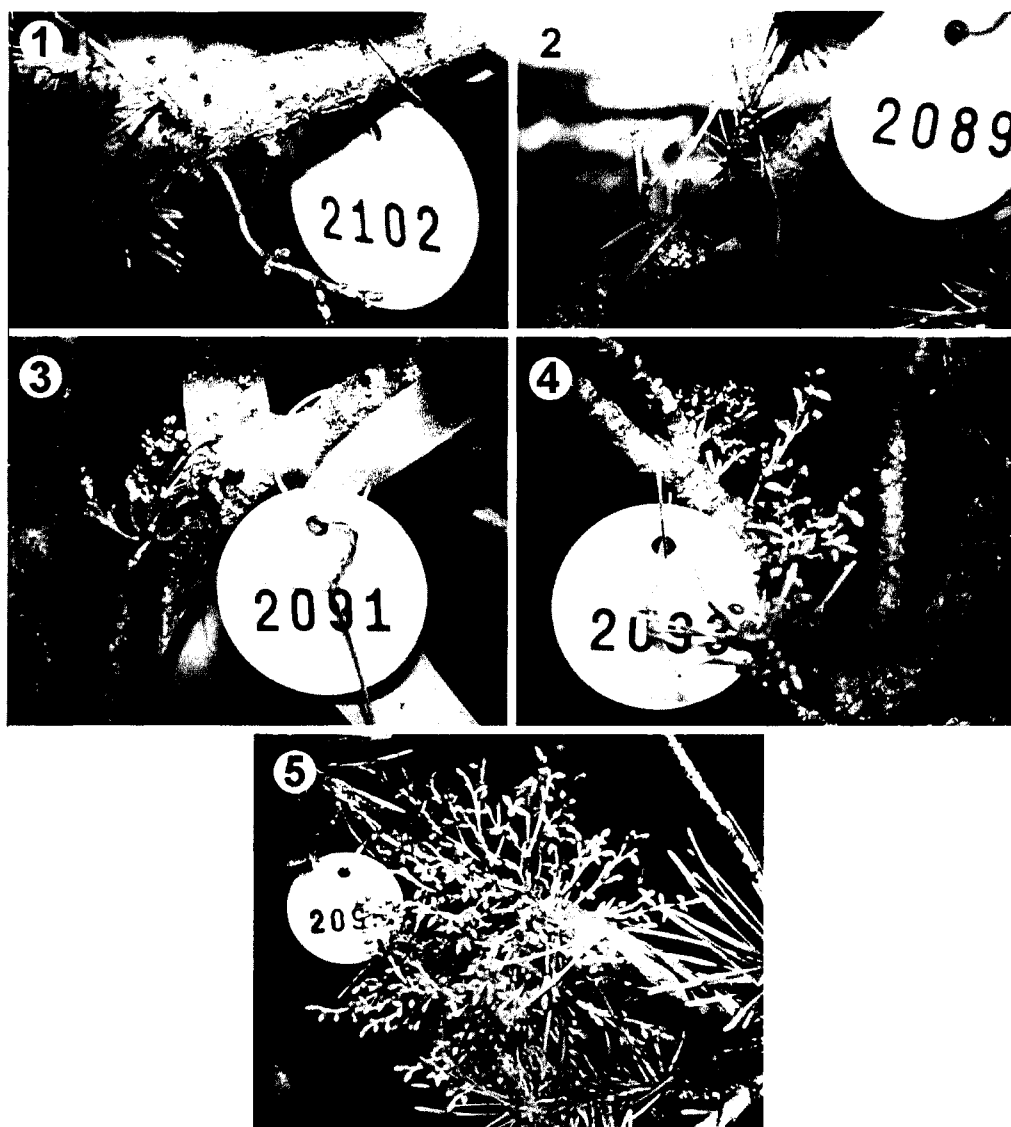


Figure 3-1. Typical *A. americanum* infections of each vigour rating. 1. Vigour rating 1. 2. Vigour rating 2. 3. Vigour rating 3. 4. Vigour rating 4. 5. Vigour rating 5.

### 3.2.5 Treatment application dates

Treatments 1, 2, 4 and 5 were applied on July 23, 2000. The weather during inoculation day was cool and overcast with a maximum temperature of 22°C at 3:19 PM, which coincided with the minimum relative humidity of 46.6%. The weather remained cool with high relative humidity for the week following inoculation (Appendix 1, 2). Treatment 6 was applied on July 25, 2000 and treatments 3 and 7 were applied on July

31, 2000. Treatment 1, but not the controls, was reapplied on August 14, 2000, three weeks after the initial inoculation, as a precaution to provide adequate inoculum.

### **3.2.6 Treatment assessment**

Following treatment, data were recorded at regular intervals. The disease rating scale developed for the greenhouse trial (Chapter 2) was utilized to assess disease progression on the treated dwarf mistletoe infections. Disease rating and vigour rating of all treatments were assessed at 2 days, 4 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 2 months, 8 months and 1 year following inoculation from the date of application of treatments 1, 2, 4 and 5. Five weeks following inoculation, *A. americanum* swelling diameter and length, distance between extreme shoots, number of shoots, number of buds, number of fruit and maximum shoot were recorded as well as disease rating and vigour rating. One year following inoculation, the same measurements and counts were performed as were taken at 5 weeks, as well as the number of female flowers. A photographic record was collected to document the treatment effect on selected replicates of the different treatments.

### **3.2.7 Sampling at one year**

One year after inoculation, a sample of 10 randomly selected (via a random number generator) replicates was collected from each of treatments 1, 2, 3, 4 and 5 and 1 replicate of treatment 6 and 7. The samples were collected and returned to the laboratory where each sample was examined thoroughly for signs and symptoms of disease. If the dwarf mistletoe shoots had any symptoms of disease, even symptoms that were not characteristic of *C. gloeosporioides*, the shoots were surface sterilized and placed on PDA. Hyphae that emerged from the diseased shoots were subcultured and *C. gloeosporioides* was identified based on cultural characteristics and spore morphology. A plate trapped antigen ELISA kit that was specific for *Colletotrichum* spp. (Adgen Identikit B, Adgen Agrifood Diagnostics, UK) was used to confirm the genus of these isolates. The manufacture's protocol was followed with one exception: 2 ml of coating buffer was introduced directly onto the mycelial mat in the petri plate, the culture was then scraped with a sterile loop and the buffer and mycelial fragments were incubated at

4°C for 1 hour followed by centrifugation at 10,000  $\times g$  for 5 minutes then 0.10 ml of the supernatant was placed in an ELISA well. The ELISA plate was incubated at 4°C overnight and then the wells were emptied and washed with the supplied wash buffer. The supplied blocking buffer was then added and the plates incubated at 37°C for one hour. Following incubation, the wells were emptied and washed and then the probe antibody (probe specificity proprietary information, Adgen Agrifood Diagnostics) was added and the plate incubated at 37°C for one hour. After washing, the conjugate was added to the wells and incubated at 37°C for one hour. A thorough wash preceded the addition of the substrate and following substrate addition, the plate was incubated in the dark at room temperature. A colour change from yellow to blue after the addition of the substrate indicated a positive reaction. The colour change was quantified by absorbance at 650 nm using a spectrophotometer that was blanked with the negative control.

### 3.2.8 Statistical analysis

Analysis of variance on ranks was used to determine if significant differences existed between treatments 1, 2, 3, 4 and 5 and Dunn's method was used to reveal which treatments were significantly different. Treatment 2 results at one year were excluded from all analyses, except the number of buds and disease rating, because the treatment process, rather than *C. gloeosporioides*, caused the change in the assessed variable. The paired observation t-test or the signed rank test was utilized to determine if significant changes in replicates of each treatment occurred over the course of the experiment by comparing the pretreatment condition to 1 year following treatment application. Chi-square analysis was used to study the relationship between disease rating and bagging the dwarf mistletoe infections with clear polyethylene bags for 48 hours post inoculation. Two by two contingency tables were constructed to compare the presence or absence of *C. gloeosporioides* and the presence or absence of the plastic bag. All statistical analyses were performed using SigmaStat version 2.03 (SPSS Inc., Chicago, IL).

### 3.3 Results

#### 3.3.1 'Stabileze' formulation

The formulation technique utilized in this field trial was the 'Stabileze' method developed by Quimby et al. (1999). This method was simple to perform and resulted in aggregates that ranged in size, with a maximum size of 5 mm. When placed on PDA, the aggregates rapidly became hydrated and *C. gloeosporioides* hyphae emerged within 24 hours. When suspended in water as a 1% solution, the formulation dissolved fully and was easily sprayed through a hand atomizer without clogging.

#### 3.3.2 Establishment of *C. gloeosporioides*

Four days following inoculation, 5 replicates of treatment 1 were showing disease, as were 3 replicates of treatment 4 and 2 replicates of treatment 5. One month following inoculation, one sample from treatment 1 was collected and *C. gloeosporioides* was recovered from the diseased tissue. Initially, brown necrotic regions were observed, which enlarged over time and girdled the shoot, resulting in death of the distal portion of the shoot. The formation of acervuli following inoculation was not observed. The result of successful establishment on *A. americanum* inoculated with *C. gloeosporioides* via treatment 1 is documented in figure 3-2. When treatment 1 was applied to the *A. americanum* shoots, many of the replicates that had symptoms of *C. gloeosporioides* disease were only showing symptoms on 1 or 2 shoots. This suggests that when the fungus became established, infection was not uniform. When the experiment was assessed one year after inoculation, 14 replicates of treatment 1 had less disease than they had at 2 months, while 7 had increased. The decrease in disease is likely due to abscission of the dead shoots.

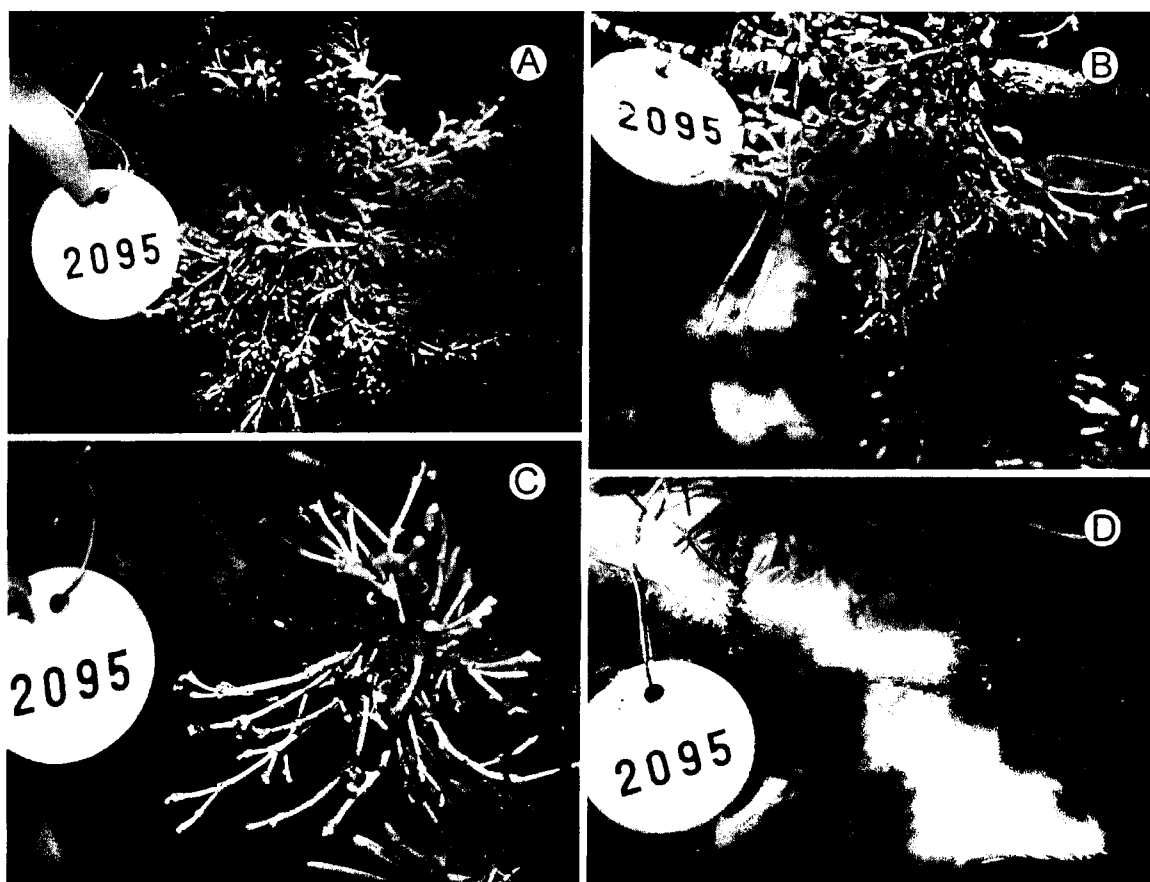


Figure 3-2. Infection of *A. americanum* by *C. gloeosporioides* as a result of application of treatment 1, 'Stabileze' formulated *C. gloeosporioides* sprayed to run-off (DR = disease rating, VR = vigour rating). This replicate was not enclosed with a polyethylene bag. A. Pre-inoculation, DR = 0, VR = 5. B. One month post-inoculation, DR = 1, VR = 5. C. Two months post-inoculation, DR = 2, VR = 5. D. One year post-inoculation, DR = 5, VR = 0.

### 3.3.3 Cut shoot inoculation – Treatment 2

All replicates that were subjected to treatment 2, 'Stabileze' formulated *C. gloeosporioides* applied to the cut shoots, had necrosis occurring at the cut ends of the shoots 4 days after treatment, while the controls (treatment 6) were dry and desiccated at the cut ends. Three weeks following application, it was noted that many of the inoculated shoots were being abscised at the basal cup. It was also noted that some buds that were not cut during treatment, because they were too small, had started to grow, resulting in

increased vigour rating. One year after inoculation, many of the replicates were producing buds, suggesting that they were recovering from treatment.

### 3.3.4 Dead replicates one year following inoculation.

All *A. americanum* infections that were selected for inclusion in the field trial were healthy and not showing signs or symptoms of disease prior to inoculation and they were located on healthy lodgepole pine branches. One year following inoculation however, several of the replicates were lost from the experiment because the infected branch was dead as a result of self-pruning by the lodgepole pine host, therefore resulting in *A. americanum* death. Other replicates were lost from the experiment as a result of squirrel damage to the localized infection and some tags had fallen off of the host branch. Dwarf mistletoe infections that were killed as a result of host branch death or squirrel damage, or from which the tag had fallen off, were removed from the experiment and not included in the statistical analysis. The number of replicates remaining in the experiment one year after inoculation is recorded in table 3-3.

Table 3-3. Number of living dwarf *A. americanum* infections prior to treatment and one year following treatment application.

Treatment	Female		Male		Total	
	Pre	1-year	Pre	1-year	Pre	1-year
1	29	26	16	13	45	39
2	29	26	16	14	45	40
3	29	22	16	16	45	38
4	31	27	14	14	45	41
5	30	25	15	14	45	39

### 3.3.5 Effect of *C. gloeosporioides* on *A. americanum* fruit production

Prior to treatment, there was no significant difference in the number of fruit present between treatments. In 2001, ANOVA on ranks indicated that the difference between treatments 1 and 3 and the controls was not significant, but the fruit crop was lower on the treated infections than the controls. The signed rank test indicated that the

number of fruit present on treatments 1, 4 and 5 were not significantly reduced from 2000 to 2001, but the number of fruit present on treatment 3 was (Figure 3-3). Variability in the number of fruit between replicates of the same treatment was high prior to treatment and in the year following treatment (Table 3-4, Figure 3-3). The number of fruit present on a single *A. americanum* infection in 2001 was independent of the number of fruit present in 2000: ie. replicate 2095 (treatment 1) had 44 shoots and 973 fruit prior to treatment and 0 shoots and 0 fruit in 2001 while replicate 2031 (treatment 1) had 17 shoots and 38 fruit prior to treatment and 27 shoots and 566 fruit in 2001. These two replicates are indicative of the amount of variation present in *A. americanum* from year to year, as well as the variation that was observed following application of treatment 1.

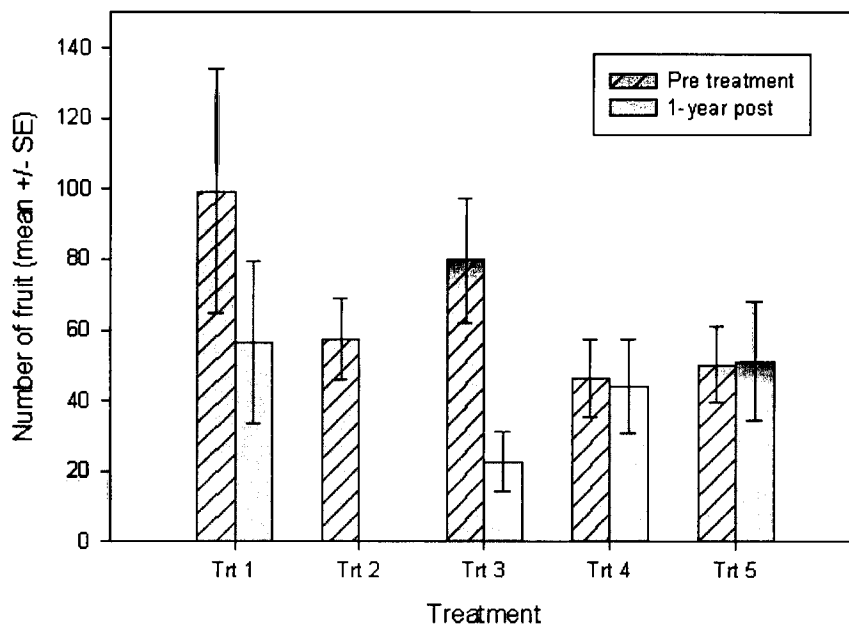


Figure 3-3. Average number of fruit per female *A. americanum* infection of each treatment prior to treatment application in 2000 and one year following application in 2001. Treatment 2 excluded from 2001 ANOVA analysis. Mean +/- standard error of the mean.

Table 3-4. Distribution of the number of fruit present on *A. americanum* infections in 2000 and 2001.

Treatment	Number of replicates with number of fruit in indicated range							
	0		1 – 10		11 – 100		101 – 1000	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	6	9	2	2	15	11	7	4
2	12	31	5	3	11	0	10	0
3	9	12	5	7	11	9	11	2
4	13	18	3	1	15	8	6	6
5	7	10	7	5	12	8	7	4

### 3.3.6 Number of female flowers

The number of female flowers present on each infection was counted one year following inoculation to provide an estimate of the number of fruit that will be present two years after inoculation. ANOVA on ranks found no significant difference between treatments. As with fruit production, flower production was highly variable amongst replicates of the same treatment.

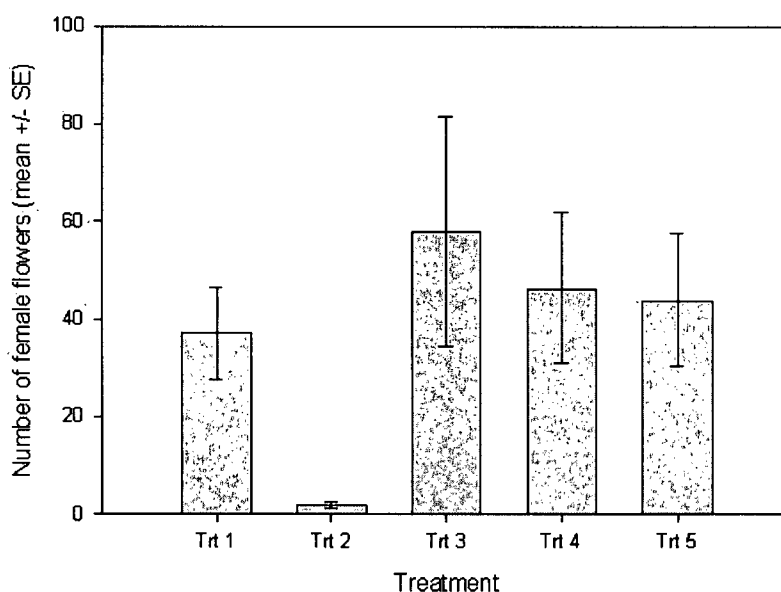


Figure 3-4. Average number of female flowers present one year after inoculation.

Treatment 2 excluded from ANOVA. Mean +/- standard error of the mean.

### 3.3.7 Effect of *C. gloeosporioides* on *A. americanum* shoot height

Analysis of maximum shoot height prior to treatment application by ANOVA on ranks indicated that there was no significant difference between the maximum shoot height of *A. americanum* infections of the different treatments. One year after treatment application, ANOVA indicated that there was no significant difference between treatments. When paired observation t-tests were conducted to compare the maximum shoot length of individual infections of each treatment in 2000 and in 2001, all treatments resulted in a significant decrease in maximum shoot height (Figure 3-5).

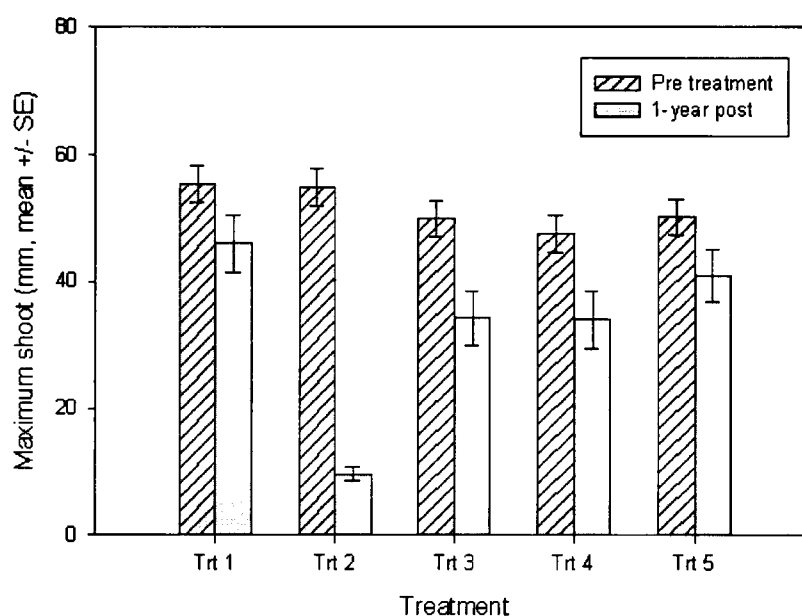


Figure 3-5. Average maximum shoot height (mm) of *A. americanum* swellings prior to treatment application in 2000 and one year following application in 2001.

Treatment 2 excluded from 2001 ANOVA. Mean +/- standard error of the mean.

### 3.3.8 Effect of *C. gloeosporioides* on the number of *A. americanum* shoots.

ANOVA on ranks indicated that there was no significant difference in the number of *A. americanum* shoots present on each infection prior to treatment or one year after treatment. The number of female shoots was decreased in 2001 from the initial number in all treatments and the signed rank test indicated that treatments 2, 3, and 4 had a significant reduction (Figure 3-6).

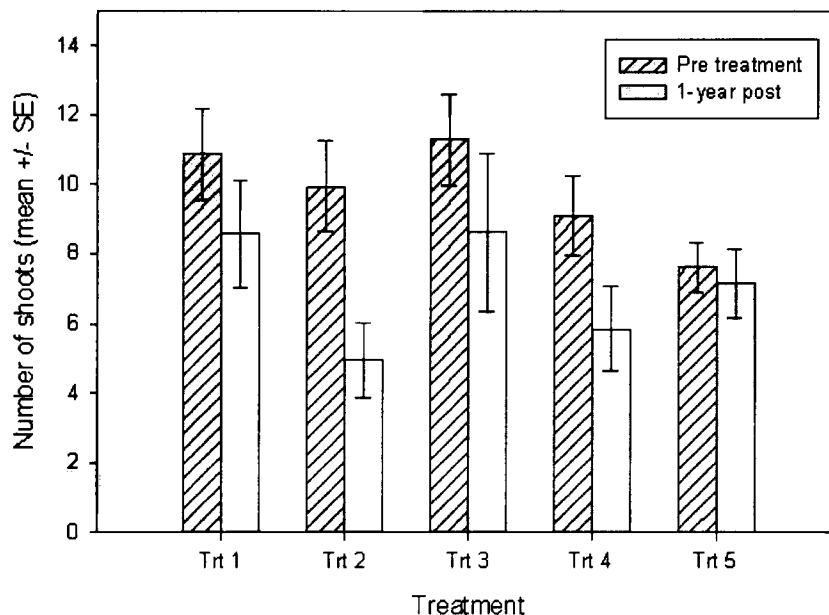


Figure 3-6. Average number of shoots per *A. americanum* infection prior to treatment application in 2000 and following inoculation in 2001. Treatment 2 excluded from ANOVA in 2001. Mean +/- standard error of the mean.

### 3.3.9 Effect of *C. gloeosporioides* on *A. americanum* bud production

ANOVA on ranks indicated that the number of buds present was not significantly different between treatments prior to treatment application or one year later. The number of buds one year following treatment was not significantly different from the number present at the time of treatment for treatments 1, 3, 4 or 5 as determined by the signed rank test. Treatment 2 however had a significant increase in the number of buds produced following treatment application, suggesting that *A. americanum* responded to the physical damage to the shoots by inducing new shoot production (Figure 3-7).

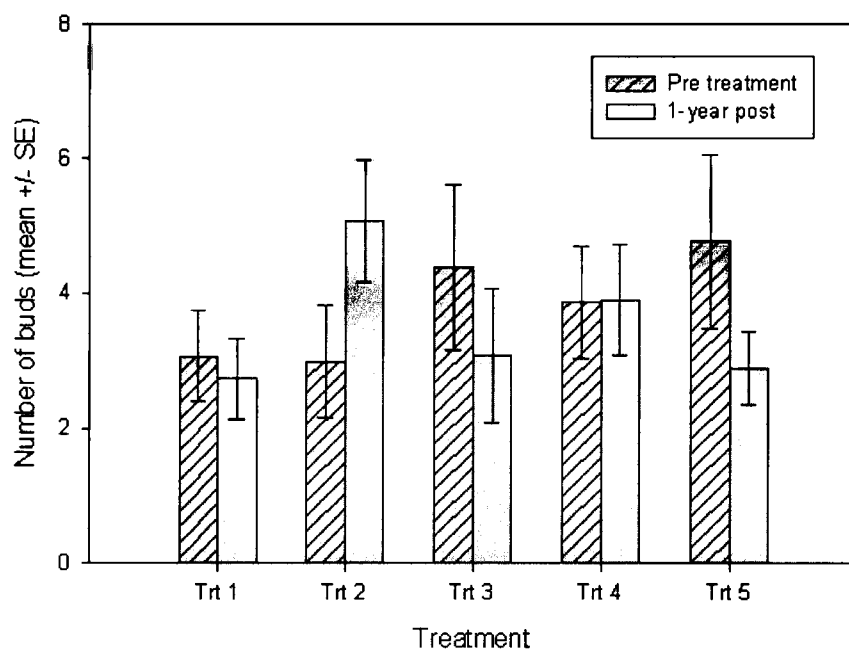


Figure 3-7. Average number of buds (shoots <5mm high) present on *A. americanum* infections prior to treatment in 2000 and one year following treatment in 2001. Treatment 2 included in ANOVA in 2001. Mean +/- standard error of the mean.

### 3.3.10 Analysis of disease rating

In addition to quantifying the change in the physical characteristics of *A. americanum*, response to *C. gloeosporioides* infection was quantified by estimation of fungal colonization of *A. americanum* shoots. Two months following inoculation, ANOVA on ranks indicated that treatments 1 and 2 had significantly higher disease ratings than treatments 3, 4 or 5 (Figure 3-8); however, one year following inoculation, the disease rating of each treatment was not significantly different between treatments (Figure 3-8). Unlike the other measures, the disease rating of *A. americanum* swellings subjected to treatment 2 was a result of fungal colonization of the cut dwarf mistletoe shoots and the associated necrosis, not an artifact of the treatment. Other than treatment 2, the disease rating was low following treatment and most of the treated *A. americanum* infections had disease ratings of 0 or 1 two months (Table 2-7), and one year (Table 2-8), following treatment application.

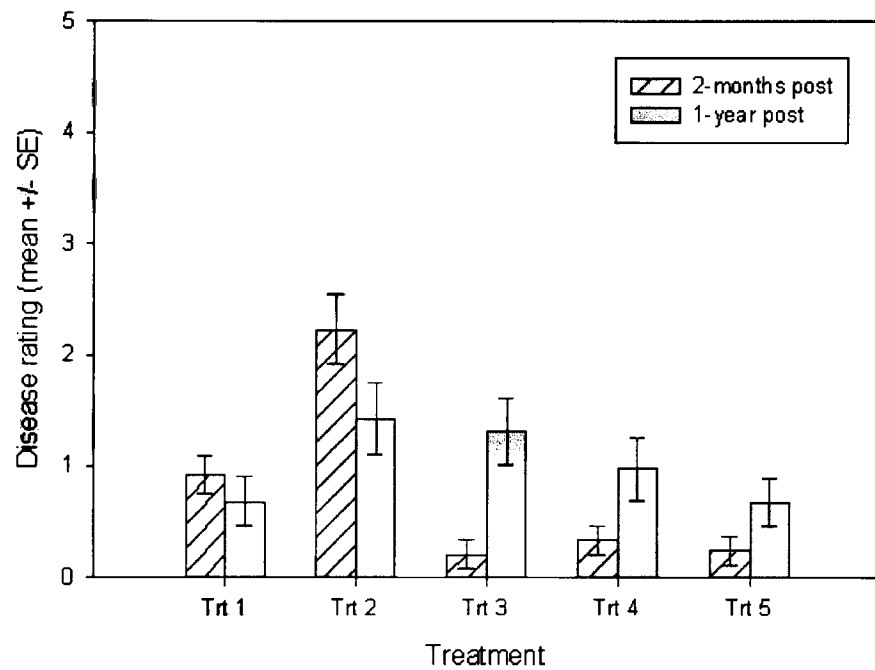


Figure 3-8. Average disease rating of *A. americanum* infections two months and one year following inoculation. Mean  $\pm$  standard error of the mean.

Table 3-5. Number of *A. americanum* infections of each disease rating (DR) for all treatments two months after inoculation.

Treatment	DR = 0	DR = 1	DR = 2	DR = 3	DR = 4	DR = 5
1	18	17	7	1	2	0
2	7	17	0	1	3	13
3	38	4	0	0	0	1
4	36	7	1	0	0	1
5	38	4	1	0	1	0

Table 3-6. Number of *A. americanum* infections of each disease rating (DR) for all treatments one year after inoculation.

Treatment	DR = 0	DR = 1	DR = 2	DR = 3	DR = 4	DR = 5
1	26	6	3	0	1	2
2	21	8	2	0	0	9
3	20	6	4	1	2	5
4	28	7	0	0	1	6
5	23	11	0	0	1	2

### 3.3.11 Vigour rating analysis

Vigour rating, a measure of the vitality of *A. americanum*, was assessed at the same time as disease rating. Prior to treatment application and at one year following inoculation, ANOVA on ranks indicated that there was no significant difference in vigour rating among treatments. When the signed rank test was used to compare individual dwarf mistletoe infections, it was found that all treatments had significantly decreased vigour ratings one year following inoculation (Figure 3-9).

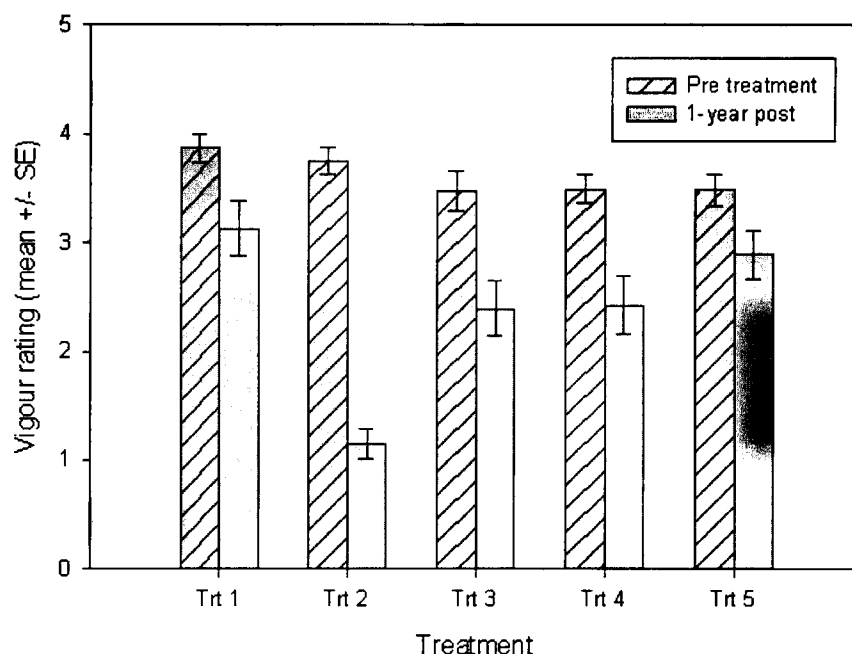


Figure 3-9. Average vigour rating of *A. americanum* prior to treatment application and at one year following application. Treatment 2 excluded from ANOVA in 2001. Mean +/- standard error of the mean.

### 3.3.12 Effect of increased humidity

The effect of artificially raising the humidity by enclosing 22 replicates of each treatment within a plastic bag for 48 hours after treatment application was analyzed using Chi-square analysis of the data from two months after inoculation when there was a significant difference in disease rating between treatments 1 and 2 and the controls. Two chi-square tests were conducted: the first included all replicates of treatments 1 through 5 ( $\chi^2 = 3.537$ ,  $df = 1$ ) while the second included only replicates of treatment 1 ( $\chi^2 = 2.681$ ,  $df = 1$ ). The results of both chi-square analyses indicated that the presence of *C. gloeosporioides* was not dependent upon the presence of the plastic bag.

### 3.3.13 Sampling in 2001

One year after inoculation, a random sample of 10 replicates of each treatment was collected from the field trial. The samples were returned to the lab where they were

screened for the presence of *C. gloeosporioides*. Of the 10 random samples collected from treatment 1, shoots of 8 were cultured, but *C. gloeosporioides* was not recovered. The basal cups and individual tissues of 2 shoots were randomly selected and cultured from all 10 samples of treatment 2 and *C. gloeosporioides* was not recovered. The branch tissue of treatment 3 was sectioned longitudinally and one half of the infection was cut at 3 to 5 mm intervals and placed on PDA. Of the 10 samples collected, *C. gloeosporioides* was recovered from 2 samples, but the fungus was restricted to the wound. ELISA confirmed the identity of the genus of this isolate as *Colletotrichum*. Three samples from treatment 4 and four samples from treatment 5 were cultured and *C. gloeosporioides* was not recovered.

### 3.4 Discussion

*Arceuthobium americanum* was inoculated with *C. gloeosporioides* under field conditions to investigate the potential of the fungus as an inundative biological control agent of *A. americanum*. The trial was installed in July 2000 and assessed at regular intervals for a year following inoculation to quantify the effect of the fungus on *A. americanum*. Field inoculation of dwarf mistletoe with *C. gloeosporioides* was conducted in the past (Parmeter et al., 1959), but no trials of the scale conducted in this study have been established.

The results of the trial suggest that the fungus did not have a significant impact on *A. americanum* and that future fruit production would not be significantly reduced as determined by flower production one year after inoculation. On some replicates *C. gloeosporioides* did become established, as shown in figure 3-1, and caused a large decrease in the reproductive ability of *A. americanum*. The fungus was isolated from the diseased tissue but the results were highly variable and a significant reduction in fruit production, compared to the controls, was not observed. One year following inoculation, there was little evidence of *C. gloeosporioides* on the inoculated swellings, likely because dead shoots were shed. The shedding of dead shoots may have prevented secondary inoculum production that would have caused further disease and increased efficacy.

There are several factors that likely contributed to the results that were observed in this field trial, including environmental conditions, inoculum concentration and isolate

selection. The maximum temperature immediately following inoculation (Appendix 1) was slightly below the optimum spore germination temperature of 25°C, but growth of the fungus should not have been inhibited by temperature. Humidity was also relatively high immediately following inoculation (Appendix 2). Length and dew period temperature are critical factors in the establishment of *C. gloeosporioides* (Luo and TeBeest, 1999). Optimum temperature and dew period requirements have been established for *Colletotrichum gloeosporioides* f. sp. *malvae* infecting round-leaved mallow (*Malva pusilla* Smith) and velvetleaf (*Abutilon theophrasti* Medic.). Makowski (1993) found that a minimum 20 hour dew period at 20°C or 25°C and 48 hours at 15°C was required to achieve 80% mortality on round-leaved mallow and 48 hours at 25°C on velvetleaf when conidia suspended in water were utilized. To establish 100% infection of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P.), a minimum dew period of 12 hours at 28°C is required by *C. gloeosporioides* f. sp. *aeschynomene* (Luo and TeBeest, 1999). When field bindweed (*Convolvulus arvensis* L.) was inoculated with *Phomopsis convolvulus* Ormeno, 18 hours of dew resulted in much better infection than short interrupted dew periods (Morin et al., 1990). In this study, *C. gloeosporioides* was formulated in a solution that provided increased humidity, and the relative humidity was high following treatment, but the number of degree hours required for successful infection and the dew period requirement may not have been satisfied. The Cascade dry cool variant of the interior Douglas-fir zone (IDF dk2), where this experiment was conducted, is characterized by a warm, dry climatic regime with a long growing season and is commonly subjected to moisture deficits (Lloyd et al., 1990). The environmental conditions that occurred during the inoculation period are likely as close to optimum for *C. gloeosporioides* colonization as can be achieved at the time of year and location where the inoculation was conducted. Formulation of *C. gloeosporioides* was conducted to compensate for the dry conditions, but it is possible that the environmental conditions that characterize this zone limit *C. gloeosporioides* establishment and that the establishment of the fungus following inoculation may be higher in moister regions. Spring or fall inoculation in the IDF dk2 may result in greater infection success than was observed in this trial due to extended dew periods, however, low night temperatures may become limiting.

Inoculum concentration is another factor that may have affected *C. gloeosporioides* establishment in this study. The 'Stabileze' formulation proved to be a very good system for delivering *C. gloeosporioides* to *A. americanum*; however, the final inoculum concentration of the fungus was approximately  $5.3 \times 10^4$  conidia per ml. This concentration of inoculum is below the concentration that causes inhibition (Morin et al., 1990), therefore establishment was not limited by a high concentration of conidia. Luo and TeBeest (1999) used an inoculum concentration of  $1.0 \times 10^5$  in their experiments with *C. gloeosporioides* f. sp. *aeschynomene* and Makowski (1993) found the best control of round-leaved mallow and velvetleaf occurred with inoculum concentrations of  $2 \times 10^6$  and  $4 \times 10^6$  spores per ml, respectively. Morin et al. (1990) suggest that high inoculum concentrations of  $1.0 \times 10^9$  conidia per m<sup>2</sup> be utilized to achieve satisfactory control under sub-optimal moisture conditions; however, as conidia concentration increases disease does not increase due to inhibition of conidia germination (Makowski 1993).

Unfortunately, experimental units of *A. americanum* that could be placed under controlled environmental conditions were not available. This prevented the detailed studies necessary to determine the exact temperature and moisture requirements, or optimum conidia concentration in the formulation, required for *C. gloeosporioides* to become established on *A. americanum*. The absence of experimental units under controlled conditions also prohibited study of the variation in virulence of *C. gloeosporioides* isolates and selection of the best isolate for field inoculation was made based on growth and sporulation characteristics in culture, not virulence assays.

The results of successful establishment on a few replicates suggest that *C. gloeosporioides* has the potential to interfere with the life cycle of *A. americanum* and thereby reduce spread and intensification of the dwarf mistletoe. Before a successful inundative biological control strategy can be utilized, several obstacles must be overcome. Further work, in a controlled environment, is required to determine the optimum conditions required for successful infection of *A. americanum* by *C. gloeosporioides* so that the formulation and application aspects of the system can be modified. It is possible that the environmental conditions of the interior Douglas fir zone are limiting and that inoculation of *A. americanum* in a different biogeoclimatic zone would have resulted in greater establishment of *C. gloeosporioides*. If the requirements

for successful establishment can be defined and further trials suggest that *C. gloeosporioides* has potential as a biological control agent, additional challenges, including techniques for inoculation of *A. americanum* infections high in the crown, must be overcome before a successful inundative biological control strategy for *A. americanum* can be utilized by the forest industry.

## **Chapter 4 – Cultural and histopathological examination of the infection of the endophytic system of *Arceuthobium americanum* by *Colletotrichum gloeosporioides*.**

### **4.1 Introduction**

Although the aerial shoots are the most obvious part of the dwarf mistletoe plant, the importance of the portion of the dwarf mistletoe growing within the host tissue, the endophytic system, cannot be overlooked. The endophytic system serves as the interface between the host and parasite. Mineral nutrients, carbon compounds and water are absorbed from the host (Alosi and Calvin, 1984), and new shoots arise from the cortical strands (Kuijt, 1955). If a fungal hyperparasite infects the endophytic system, not only will absorption of nutrients be affected, but new shoot production will be as well. From an inundative biological control standpoint, reduction of new shoot production would increase the interval between biocontrol treatment because the period of time between application and new fruit production would be extended. If the hyperparasite were able to kill the endophytic system, new shoot production, and therefore fruit production, would be eliminated. This chapter examines the role of *Colletotrichum gloeosporioides* in the endophytic system of *Arceuthobium americanum*.

The aerial shoots have direct continuity with the endophytic system of the dwarf mistletoe. The endophytic system is composed of longitudinally and tangentially oriented strands and radially oriented sinkers (Alosi and Calvin, 1984). The radially oriented sinkers become embedded in the host xylem as the vascular cambium divides to add xylem tissue (Hunt et al. 1996). As the sinker penetrates the vascular cambium, the cambial cells remain undamaged and ray formation is stimulated. The ray gradually becomes embedded as new xylem is produced by the cambium (Kuijt, 1955). This occurs as fusiform initials are converted to ray initials adjacent to the parasite cells, resulting in the formation of a radially-oriented sinker that is surrounded by host ray cells (Alosi and Calvin, 1984b). This structure has been termed an “infected ray” by Srivastava and Esau (1961), which is an appropriate term as the development of the sinker occurs in close association with the host. As the sinker becomes embedded in the xylem tissue, meristematic activity must occur so that continuity with the cortical strands

is maintained. Exactly where within the sinker this meristematic activity occurs has been debated. Cohen (1954) suggests that sinkers are initiated by meristematic parenchyma cells within the cortical strand and that growth in length of the sinkers occurs by cell division at the base of the sinker where it joins the strand. Srivastava and Esau (1961) suggest that the parenchyma cells that cross the host cambium are meristematic because they are spindle-shaped and shorter and have denser cytoplasm than sinker cells in the xylem, as well as the presence of protoplasts and groupings of derivatives of single cells. Alosi and Carol (1984b) have observed distinct intercalary meristems juxtaposed to the host cambium, but they have also observed cellular arrangement within the neck of sinkers that suggests meristematic activity. It has been observed that many sinkers have no, or discontinuous, xylem and that no direct interspecific tracheary element connections exist. Water and mineral uptake by the mistletoe occurs via continuity of host and parasite cellulosic cell walls and through specialized half-bordered pits that are adjacent to sinker cells. It is hypothesized that water flows into the sinker and eventually to xylem in the cortical strand, where there is low resistance to movement and negative water potential created by transpiration in the aerial shoots results in continual water supply (Alosi and Carol, 1984).

The aerial shoots and sinkers are connected by the cortical strands, which are located within the host phelloderm, cortex and phloem (Hunt et al., 1996). The strands are predominantly longitudinal and they cross and anastomose, as indicated by the presence of several steles within a single strand (Cohen, 1954). The sinkers are usually connected to the cortical strand by parenchyma cells, as xylem is typically not present in the sinker. Cortical strands have a central xylem core that is surrounded by a sheath of parenchymous cells (Hunt et al., 1996). Extension of the cortical strand occurs by an apical cell that divides transversely to create a row of segments, which divide longitudinally. These cells then undergo anticlinal and periclinal divisions to create longer strands of increased diameter. The periclinal divisions result in an inner core of small cells that are surrounded by larger cells. The small cells are meristematic and differentiate into xylem tracheary elements (Bhandari and Nanda, 1970). There is no phloem in the cortical strand (Cohen, 1954). The production of new shoots from the cortical strand occurs by the production of buds that appear as knob-like meristematic

protuberances on the outer cortical strand. As the bud develops, the basal cells divide and develop an intercalary stalk with tiered cells. When the bud is pushed to the surface, a cork cambium is formed by the host to produce a periderm that protects the cortical parenchyma cells of the host (Cohen, 1954).

As outlined above, the aerial shoots are directly connected to the endophytic system within the host tissue and new shoots arise from the cortical strands. The connectivity between the aerial shoots and the endophytic system provides a conduit through which *C. gloeosporioides* may be able to infect the endophytic system of *A. americanum*. *Colletotrichum gloeosporioides* infection is initiated when a conidia, aided by the conidial matrix, is adhered to susceptible plant tissue. A melanized appressorium is produced at the terminal end of the germ tube after conidial germination, from which arises a penetration peg that penetrates the cuticle. After penetration of the cuticle, *C. gloeosporioides* hyphae ramify through the tissue during the symptomless biotrophic phase of infection and then secondary hyphae are formed that cause necrosis during the necrotic stage of infection (Bailey et al., 1992). If the endophytic system did become diseased as a result of *C. gloeosporioides* travelling from the shoots into the endophytic system, production of new shoots may be affected because new shoots arise from the cortical strands, which would effect the treatment periodicity of a biological control agent.

The role of *C. gloeosporioides* within the endophytic system of *Arceuthobium campylopodum* Engelm. f. *abietinum* (Engelm.) Gill parasitizing red fir (*Abies magnifica* Murr) was investigated by Parmeter et al. (1959). The authors noted that when placed on water agar, superficial acervuli with setae developed in the region of the cortical strands of the endophytic system. The authors sectioned the fir bark and wood and were able to observe mycelium in the endophytic system. Diseased dwarf mistletoe infections were collected and *C. gloeosporioides* was isolated from the woody tissue of 12 of the 27 samples. In a second study, *C. gloeosporioides* was observed and isolated from the woody tissue of 5 of 5 diseased dwarf mistletoe swellings. From these observations, it was concluded that *C. gloeosporioides* was able to infect the endophytic system of *A. campylopodum* f. *abietinum* (Parmeter et al., 1959). Wicker and Shaw (1968) collected *C. gloeosporioides* from *A. americanum*, *A. campylopodum*, *A. laricis* and *A. douglasii*

and observed *C. gloeosporioides* hyphae penetrating the “interior cortical region, but never the vascular elements”, but it is unclear whether the fungus was observed in the endophytic system of all these species.

From a biological control standpoint, the results reported by Parmeter et al. (1959) and the observations of Wicker and Shaw (1968) are encouraging. They concluded that the endophytic system was colonized by *C. gloeosporioides*. However, many details were omitted. It is not stated precisely where within the endophytic system the fungus was observed and the pathological effects of the fungus in the endophytic system were not described. The objectives of this study were to determine if *C. gloeosporioides* was able to infect the endophytic system of *A. americanum* and to describe the pathological effects of infection.

## **4.2 Materials and Methods**

### **4.2.1 *A. americanum* naturally infected with *C. gloeosporioides***

Diseased and healthy localized *A. americanum* infections were collected in the summer of 2000 from Logan Lake and Lytton, BC. Dwarf mistletoe infections that were showing extensive signs of disease, and were located on otherwise healthy branches, were split longitudinally and one half was surface sterilized with 95% ethanol and 10% sodium hypochlorite and the other half was preserved in formalin acetic acid (FAA) (Johansen, 1940). Samples were aseptically removed from the surface sterilized dwarf mistletoe infections and they were carefully dissected into small chips to isolate tissue from xylem, cambium, phloem, phellum, and the basal cups. The individual tissue types were then plated on potato dextrose agar (PDA, Difco), incubated at 20°C and observed at regular intervals. Fixation and isolation was conducted immediately after collection from the field. If *C. gloeosporioides* was isolated from any of the tissues sampled, the sample was selected for further microscopic investigation. Shoot sections with signs of disease were also surface sterilized and plated out to isolate *C. gloeosporioides* and preserved in FAA so that later microscopic examination could be conducted to observe *C. gloeosporioides* damage to the aerial tissues. Healthy *A. americanum* swellings were collected and processed in the same manner as the diseased samples to verify that the

sample was free of *C. gloeosporioides* and to provide uninfected checks for comparison with diseased material.

#### **4.2.2 Artificial infection of *A. americanum* with *C. gloeosporioides***

Two of the treatments applied in the field trial (Chapter 3) were designed to introduce *C. gloeosporioides* into the endophytic system of *A. americanum*. Treatment 2 involved placing 'Stabileze' formulated *C. gloeosporioides* directly onto the cut ends of shoots that were cut approximately 5 mm from the branch. Treatment 3 was applied by artificially wounding the dwarf mistletoe infection with a sterile cork borer, applying a mycelial plug of *C. gloeosporioides* to the cambium and wrapping the wound with Parafilm M. Inoculation of cut dwarf mistletoe shoots with sterile formulation and inoculation of an artificial wound with sterile PDA were controls for these treatments. In the summer of 2001, approximately one year after inoculation, 10 of the 45 replicates of each treatment were randomly selected and harvested to determine if *C. gloeosporioides* had colonized the endophytic system of *A. americanum*. To compare the inoculated treatments with the controls, 1 of the 5 replicates of each control treatment was also collected.

At the laboratory, the sample dwarf mistletoe infections of each treatment were examined closely under a dissecting microscope to identify signs and symptoms of *C. gloeosporioides* infection. After observation, the infections were sectioned longitudinally and one half of the infection was fixed in FAA and preserved in 70% ethanol and the other half was cultured. Basal cups of two shoots were randomly selected on the infections subjected to treatment 2, and its control, and the basal cups as well as all tissue layers below the basal cup were aseptically removed from the infection. The tissue layers were then separated and plated independently on PDA to determine how deeply *C. gloeosporioides* was able to penetrate the endophytic system. Dwarf mistletoe infections of treatment 3 were cut at 3 to 5 mm intervals, centered on the middle of the inoculation wound, and the pieces were plated on PDA to determine the distance that *C. gloeosporioides* had traveled from the wound. The control for treatment 3 was processed in the same manner as the inoculated swellings. The PDA plates were incubated in the dark at 20°C and checked regularly for the presence of *C. gloeosporioides*.

#### 4.2.3 Paraffin embedding

Samples from which *C. gloeosporioides* was isolated, check samples that were not infected and *C. gloeosporioides* infected *A. americanum* shoots were prepared for microscopic examination. The samples were fixed in FAA for 48 hours and then transferred to 70% ethanol for long term storage. After the presence of *C. gloeosporioides* was confirmed by culturing (or confirmed to be absent in the case of the check), three small pie shaped pieces were cut from the diseased swelling tissue. Three pieces were removed from each sample to allow radial, tangential and longitudinal sections to be cut. The samples were then taken through an alcohol dehydration process to remove water from the samples and then embedded in paraffin wax (Paraplast+, Oxford Labware, St. Louis, MO). The pieces were dehydrated by transferring from 70% ethanol to 70% TBEA (TBEA = 75% 2-methylpropan-2-ol tert-butanol, 25% absolute ethanol) overnight, then 70% TBEA to 85% TBEA for the day, followed by 85% TBEA to 95% TBEA overnight, then 95% TBEA to 100% TBEA for the day and 100% TBA (2-methylpropan-2-ol tert-butanol) overnight. The paraffin embedding procedure was initiated by transferring from 100% TBA to TBA:paraffin oil (Sigma, St. Louis, MO) 1:1 for 8 hours, then pouring the pieces and liquid TBA:paraffin solution onto solidified Paraplast+ and placing the dish in a 60°C oven to melt. Once melted, the pieces dropped into the Paraplast+ and the liquid was poured out of the dish and pure Paraplast+ was poured in. The dish was then placed in the oven at 60°C under vacuum. Over a period of five days, the Paraplast+ was changed three times per day and the vacuum was gradually increased until 762 mm Hg was reached and bubbles stopped emerging from the pieces. Once the Paraplast+ was fully infused into the sample piece, the piece was embedded by placing the sample, with the side to be sectioned down, into molten Paraplast+ and allowing the Paraplast+ to solidify.

After embedding, the pieces were mounted on a microtome object holder and the wax was removed down to the surface of the sample. The sample was then placed in a solution of molliflex (BDH, Toronto, ON):Ethylene glycol (FisherBrand, Pittsburgh, PA) 1:1 at room temperature for five to seven days to soften the sample prior to sectioning. After softening, 10 micron thick sections were cut with a rotary microtome (American Optical, model 820). The samples were returned to the softening solution when the

samples became too hard to section and were again sectioned after a further five to seven days in the softening solution. This cycle was repeated until adequate sections of the sample piece had been made.

The sections were mounted on slides by floating a ribbon of sections upon a puddle of 4% formalin that was laid over a slide that had been pretreated with Haupts adhesive (Johansen, 1940). The formalin was evaporated and the sections bound to the slide by placing the slide on a hotplate at 50°C. Once the sample ribbons were bound to the slide, they were ready for staining.

#### **4.2.4 Staining**

Three staining methods (safranin – picro=aniline blue, Johansen's safranin and rhodamine B – methyl green; Appendix 3) were tested to determine which method provided the best differentiation between fungal and plant material. Safranin – Picro=aniline blue proved to be superior and it was used for all the tissue staining in this chapter.

#### **4.2.5 Observation and photomicrography**

After staining, Permunt (Fisher Chemical Co., Fair Lawn, NJ) was used to adhere a coverslip to the stained sections. Once the permunt had set, slides were examined under the light microscope and observed at magnifications up to 400x using a Nikon Optiphot-2 microscope fitted with a phase contrast adapter or a Zeiss Photomicroscope-II fitted with a polarizing filter (Zeiss 47 36 00). Both microscopes had camera attachments and photomicrographs were taken using Kodak Elitechrome 160T tungsten film (Eastman Kodak Co., Rochester, NY).

### **4.3 Results**

#### **4.3.1 Culturing and field observation of *C. gloeosporioides* inoculated *A. americanum*.**

##### **4.3.1.1 *A. americanum* naturally infected with *C. gloeosporioides***

*Arceuthobium americanum* infections that were located on otherwise healthy branches, yet had no shoots and appeared to be diseased, were collected and returned to

the lab where the woody tissues were dissected, aseptically removed and cultured. *Colletotrichum gloeosporioides* was isolated from the basal cup region of 8 of the 17 *A. americanum* infections collected. The fungus was never isolated from the inner bark tissues or xylem. Other fungi, including *Cladosporium* sp. and *Sclerophoma pithyophila* were commonly isolated from the outer bark tissues, but not the inner bark or xylem. Culturing the woody tissues from healthy *A. americanum* swellings resulted in isolation of *Cladosporium* sp. from 1 swelling. *Colletotrichum gloeosporioides* was not isolated from the woody tissues of healthy *A. americanum* swellings.

To investigate the role of *C. gloeosporioides* as an endophyte, healthy *A. americanum* shoots adjacent to diseased shoots, and healthy portions of diseased *A. americanum* shoots, were surface sterilized and plated. *Colletotrichum gloeosporioides* was only isolated from the diseased portions of the *A. americanum* shoots, not the healthy tissues, suggesting that it does not act as an endophyte.

#### **4.3.1.2 Artificial infection of *A. americanum* with *C. gloeosporioides***

Inoculation of the cut *A. americanum* shoots with 'Stabileze' formulated *C. gloeosporioides* resulted in necrosis that began at the cut surface and traveled down the shoot. One month after inoculation 100% shoot abscission had occurred on 27 of 45 replicates of treatment 2, while 1 of 5 replicates of the control had 0 shoots. One year after inoculation, 5 infections that were subjected to treatment 2 were dead as a result of the host branch death. Of the 40 remaining replicates, 24 had shoots and buds, 8 had buds but no shoots, 3 had shoots but no buds and 5 had neither shoots nor buds. Coincidentally, 2 of the 5 replicates with no shoots or buds (2183 and 2135) were collected during the random sampling and cultured. Shoots and buds were present on all of the control replicates at 1-year.

Culturing the individual tissue types directly below the basal cups of 2 shoots of each of the 10 randomly selected replicates that were subjected to treatment 2 did not result in the recovery of any *C. gloeosporioides* isolates. Other fungi, including *Sclerophoma pithyophila* and *Cladosporium* sp. were recovered and every replicate had at least one fungus present. Plating the individual tissue types of the control for treatment

2 resulted in isolation of *Sclerophoma pithyophila* but not *Cladosporium* sp. or *C. gloeosporioides* (Table 4-1).

Treatment 3 was an alternative method of inoculating the endophytic system with *C. gloeosporioides*. Immediately after wounding, the wound filled with copious quantities of pitch. Every replicate of treatment 3 that was cultured had the same fungal species as treatment 2 and *Colletotrichum gloeosporioides* was recovered from the inoculation site of 2 replicates. Both *Sclerophoma pithyophila* and *Cladosporium* sp. colonized the control for treatment 3 (Table 4-1).

Table 4-1. Recovery of fungi in culture from *A. americanum* subjected to treatment 2 and 3 during the field trial. A total of 10 replicates of the treatment and 1 of the control were randomly selected.

Treatment	Fungus	# of replicates infected	% infection of sample
Treatment 2	<i>Sclerophoma pithyophila</i>	7	70
	<i>Cladosporium sp.</i>	1	10
	<i>Colletotrichum gloeosporioides</i>	0	0
	Other	4	40
Control for treatment 2	<i>Sclerophoma pithyophila</i>	1	100
	<i>Cladosporium sp.</i>	0	0
	<i>Colletotrichum gloeosporioides</i>	0	0
	Other	0	0
Treatment 3	<i>Sclerophoma pithyophila</i>	10	100
	<i>Cladosporium sp.</i>	2	20
	<i>Colletotrichum gloeosporioides</i>	2	20
	Other	4	40
Control for treatment 3	<i>Sclerophoma pithyophila</i>	1	100
	<i>Cladosporium sp.</i>	1	100
	<i>Colletotrichum gloeosporioides</i>	0	0
	Other	0	0

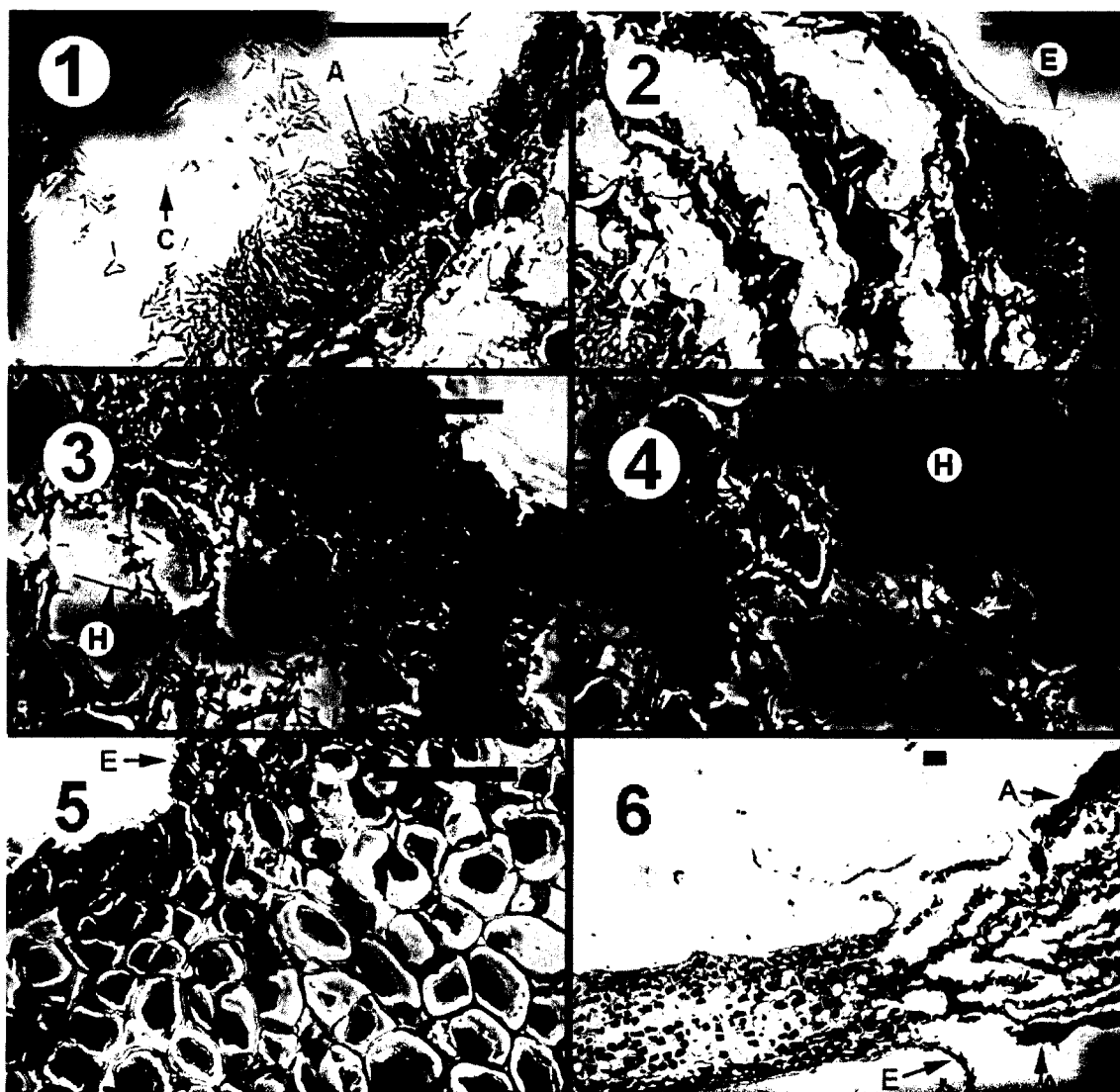
#### 4.3.2 Histopathological examination

##### 4.3.2.1 *C. gloeosporioides* infected *A. americanum* shoots

Longitudinal and cross sections of diseased *A. americanum* shoots revealed that the epidermal layer of the shoot was blistered open to allow conidia to be released from acervuli. The acervuli did not contain setae and the acervulus was integrated into the mesophyll cells of the shoot and hyphae of *C. gloeosporioides* were observed to penetrate the shoot to the xylem. The mesophyll cells of the infected portion of the shoot were collapsed and unorganized, and intercellular and intracellular hyphae were observed. The boundary between healthy and necrotic shoot tissue was readily observed (Figure 4-1).

Figure 4-1. *Colletotrichum gloeosporioides* infected *Arceuthobium americanum* shoots.

Safranin – Picro=analine stain. Scale bars represent 100  $\mu\text{m}$ . 1. Acervulus (A) of *C. gloeosporioides* producing conidia (C). Longitudinal section. 2. Acervulus of *C. gloeosporioides*. Cross section. Note: hyphae present in the center of the shoot at the xylem core. Epidermis (E), xylem (X). 3. Acervulus of *C. gloeosporioides* prior to breaking through the epidermal layer. Acervuli is integrated with host mesophyll cells. Hypha (H). 4. Longitudinal section of diseased shoot with intercellular hyphae (H) of *C. gloeosporioides*. Cells are disrupted and not organized. 5. Longitudinal section of healthy *A. americanum* shoot tissue with healthy, ordered mesophyll cells. Epidermis (E). 6. Diseased and healthy *A. americanum* tissue, showing the extent of necrosis. Acervuli (A) are present on the upper and lower shoot surfaces, close to the node (node is not in micrograph). Epidermis (E).



#### **4.3.2.2 *C. gloeosporioides* infected *A. americanum* parasitizing *P. contorta* var. *latifolia* branch tissue**

Hyphae with the same morphology and staining characteristics of *C. gloeosporioides* in diseased shoots were observed in the dead outer bark tissues and associated with basal cups of *A. americanum*. A second hyphal type that was observed appeared to be melanized and it penetrated into the dead outer bark, and was present on the bark surface. Small circular cells were observed in association with this hyphal type, suggesting that the fungus was sporulating on the bark surface (Figure 4-2).

The endophytic system of *A. americanum* was readily observed in the lodgepole pine xylem and bark tissues; however, no fungal hyphae were associated with these tissues. Xylem cells were observed in all parts of the endophytic system, including the sinkers, however, the xylem tissue observed in the sinkers are lodgepole pine ray tracheids as determined by the prominent and complex "dentate" cell wall thickenings (Koch, 1996). The xylem tissue appeared to be discontinuous and scattered throughout the sinker, rather than arrayed into a xylem column. Xylem tissue arrangement observed in longitudinal and cross sections of the cortical strands was also unorganized, however, it appeared that continuous xylem columns were present (Figure 4-3).

Figure 4-2. Transverse sections of healthy and *C. gloeosporioides* infected *A.*

*americanum* tissues on and in lodgepole pine bark. Scale bars represents 100  $\mu\text{m}$ . 1. Spores (S) of melanized hyphae, on the surface of the bark? 2. Melanized hyphae (MH) on bark surface. 3. Melanized hyphae outside of bark tissue. 4. Melanized hyphae (MH) penetrating into the outer bark tissue. 5. Putative intercellular *C. gloeosporioides* hyphae (Cg H) present near bark surface. 6. *C. gloeosporioides* (Cg H) and melanized hyphae (MH) present in the outer bark tissue of lodgepole pine. 7. *A. americanum* shoot base with *C. gloeosporioides* hyphae present between shoot base and bark (arrow). 8. *C. gloeosporioides* hyphae (Cg H) in region shown in plate G.

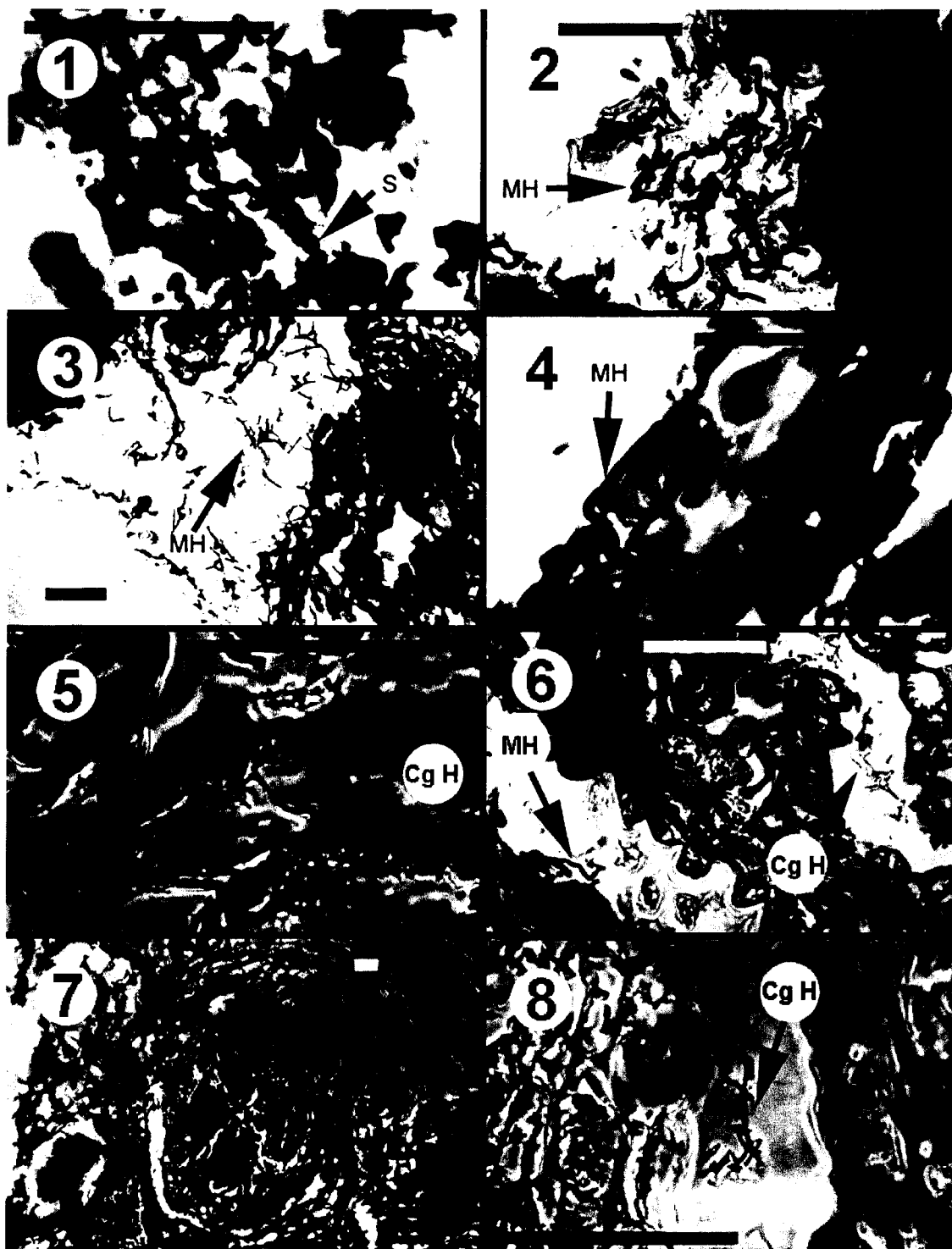
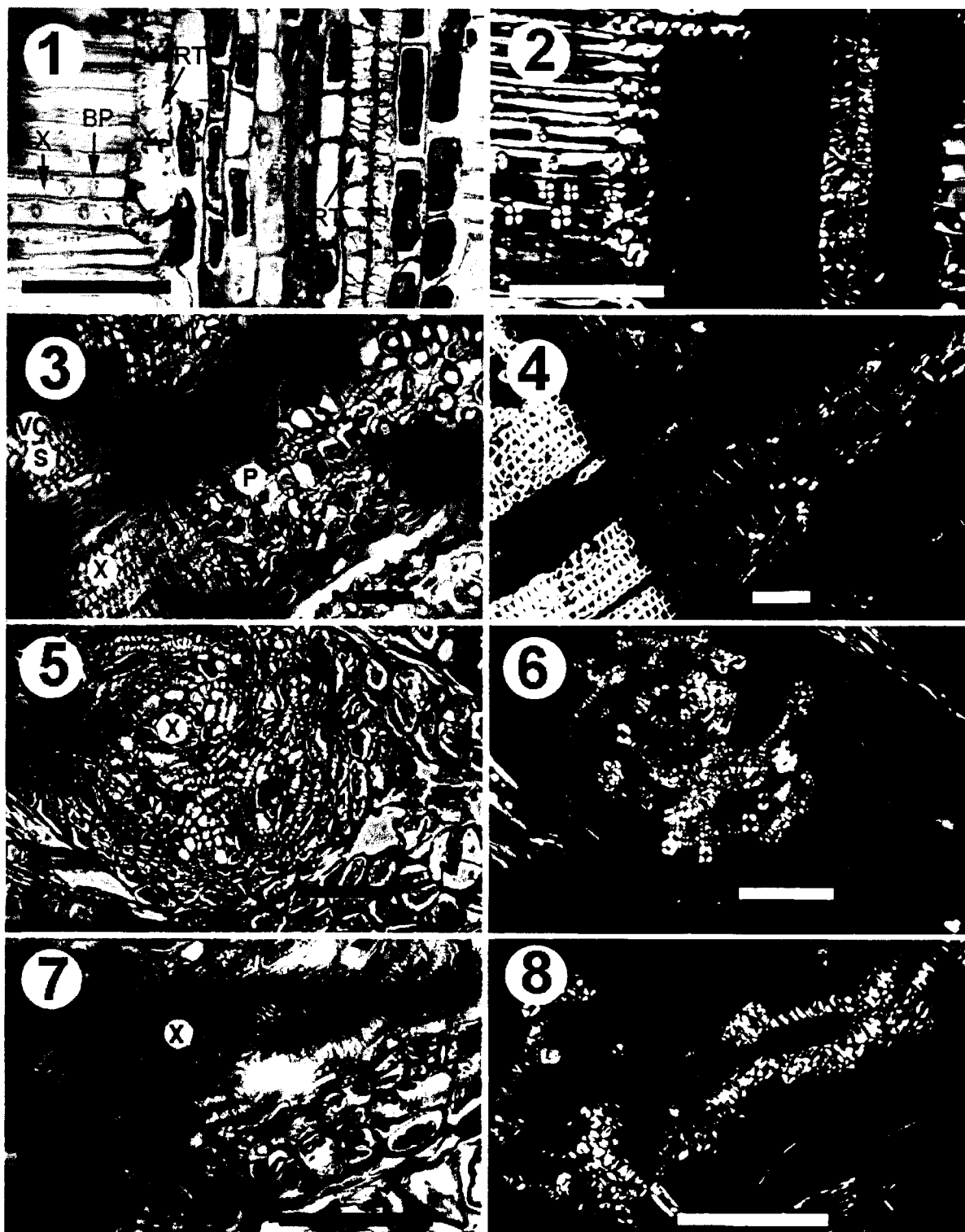


Figure 4-3. Xylem cells in various endophytic tissues of *Arceuthobium americanum*.

Pair of micrographs in transmitted light and birefringence to show xylem cells.

Scale bar represents 100  $\mu\text{m}$ .

- 1, 2. Long section of sinker in host xylem. Micrograph is oriented horizontally. X=host xylem, BP=borderd pit, RT=ray trachied.
- 3, 4. Cross section of sinker crossing the vascular cambium. S=sinker, VC=vascular cambium, P=phloem, X=xylem.
- 5, 6. Cross section of a radial cortical strand in a longitudinal section of bark. X=*A. americanum* xylem.
- 7, 8. Longitudinal section of a cortical strand in a longitudinal section of bark. X=*A. americanum* xylem.



#### 4.3.2.3 *C. gloeosporioides* inoculated *A. americanum*.

The control and the two infections from which *C. gloeosporioides* was recovered after inoculation of the cut shoots were embedded in paraffin, sectioned and stained. No fungal hyphae were observed in the endophytic system or any of the host tissues; however, hyphae were observed on the bark surface. The hyphae appeared melanized and did not match the *C. gloeosporioides* hyphae observed under acervuli in the shoots or in the bark of the naturally infected *A. americanum* infections that were described above.

### 4.4 Discussion

Infection of the endophytic system of *Arceuthobium campylopodum* f. *abietinum* parasitizing red fir by *C. gloeosporioides* was observed by Parmeter et al. (1959) and Wicker and Shaw (1968) observed *C. gloeosporioides* infection of the endophytic system of *A. americanum*, *A. campylopodum*, *A. laricis* and *A. douglasii* (assumed because it is not clearly stated exactly which species were colonized). Infection of the endophytic system of *A. americanum* by *C. gloeosporioides* during biological control treatment would likely result in a decrease in the frequency of biological control application required to manage dwarf mistletoe because new shoot production would be reduced. Investigation of the role of *C. gloeosporioides* in the endophytic system of *A. americanum* was undertaken to provide a better understanding of the role of the fungus in this pathosystem.

#### 4.4.1 Culturing

The results of culturing the woody tissues of naturally infected *A. americanum* infections indicated that *C. gloeosporioides* was restricted to the basal cup and dead outer bark tissues. Inoculation of cut *A. americanum* shoots with *C. gloeosporioides* did not result in infection of the endophytic system. Other fungi, including *Sclerophoma pithyophila* and *Cladosporium* sp. were commonly isolated from the outer dead bark of *A. americanum* swellings that were subjected to treatment 2. Field observation of the response of cut *A. americanum* shoots to inoculation suggests that it is likely that the shoots were abscised from the dwarf mistletoe infection prior to *C. gloeosporioides* reaching the endophytic system via the inoculated shoots.

*Colletotrichum gloeosporioides* was recovered from the woody tissue of lodgepole pine one year following *C. gloeosporioides* inoculation of an artificial wound in the lodgepole pine bark at the center of localized *A. americanum* infection, but the fungus was restricted to the inoculation point. Copious pitch flooding into the wound was likely inhibitory to *C. gloeosporioides* through the creation of an anerobic environment, or toxicity or both, thereby compartmentalizing the fungus and preventing further spread.

#### 4.4.2 Histopathology

Conidia production from acervuli on the diseased shoots was copious, but no setae were observed in association with the acervuli on *A. americanum* shoots. Setae were not observed on *C. gloeosporioides* infected *A. campylopodum* f. *abietinum* shoots by Parmeter et al. (1959); however, setae production was recorded on *C. gloeosporioides* infected *A. campylopodum* shoots (Wicker, 1967) and shoots of *A. americanum*, *A. laricis* and *A. douglasii* (Wicker and Shaw, 1968). It is possible that the differences in setae production were due to different races of *C. gloeosporioides* on the different *Arceuthobium* species.

The presence of intracellular hyphae and hyphal penetration to the shoot xylem (Figure 4-1, panel B) observed in this study may represent a later stage in the infection process than was observed by Wicker and Shaw (1968) who found only intercellular hyphae that were limited to the exterior of the shoot cortex. In this study, destruction of *A. americanum* mesophyll cells in the aerial shoot as a result of the necrotic phase of *C. gloeosporioides* infection was clearly evident in the infected tissues. *Colletotrichum gloeosporioides* was not recovered from, or observed in, the endophytic system of *A. americanum* or the woody tissue of lodgepole pine; it was restricted to the aerial portion of *A. americanum* and the basal cup region of the swelling. These results are contrary to the observations of Parmeter et al. (1959) and Wicker and Shaw (1968) and may be because a different dwarf mistletoe / host association was studied here, or differences in the behaviour of *C. gloeosporioides* due to genetic variation of the fungus. It is also possible that if the sample size of this study was increased, *C. gloeosporioides* may have been observed in the endophytic system of *A. americanum*.

#### 4.4.3 Potential *C. gloeosporioides* exclusion mechanism

A combination of factors of the host and pathogen may be responsible for the fact that *C. gloeosporioides* was not observed to infect the endophytic system in this study. In order for the endophytic system to become diseased, the fungal hyphae must travel from the infection initiation point into the endophytic system. Continuity of the shoot with the endophytic system was observed in the histopathology portion of the study, providing a route for *C. gloeosporioides* to infect the endophytic system. During the necrotic phase of the disease cycle, diseased tissue containing hyphae was clearly distinguished from the healthy tissue, and fungal hyphae were not observed in healthy tissue. Necrosis was observed traveling down the shoot from the inoculation point to the shoot base, however, the diseased shoots were abscised from the dwarf mistletoe swelling and shed as the infection process progressed. This suggests that *A. americanum* prevented infection of the endophytic system by shedding diseased shoots.

Infection of the endophytic system is not required for completion of the life cycle of *C. gloeosporioides*; conidia infect the aerial shoots and new conidia are produced in acervuli located on the shoots. Pectin lyase and polygalacturonase are produced during the infection process (Bailey et al., 1992; Senaratna et al., 1991), which would likely result in degradation of the parenchymous cells of the endophytic system but *C. gloeosporioides* is able to complete its life cycle in the shoot tissue, obviating the need for parasitism of the endophytic system.

#### 4.4.4 Conclusion

*Colletotrichum gloeosporioides* has not been reported to infect lodgepole pine and culturing of diseased *A. americanum* swellings resulted in isolation of the fungus from diseased portions of the shoots and the basal cup region only; however, several different fungi were isolated and observed on the dead outer bark tissue of *P. contorta* var. *latifolia*. The previous reports of *C. gloeosporioides* in the endophytic system are vague and refer to *C. gloeosporioides* exclusively (Parmeter et al., 1959; Wicker and Shaw, 1968). The results of this study indicate that many different fungi are associated with the dead outer bark tissues of *P. contorta* var. *latifolia* infected by *A. americanum*. The observations of this research suggest that infection of *A. americanum* with *C.*

*gloeosporioides* resulted in a decrease in the number of shoots on the dwarf mistletoe infection through shoot abscission, but that it had no effect on the production of new shoots because the endophytic system was not infected.

#### **4.4.5 Future research**

The evidence presented in this research suggests that *C. gloeosporioides* does not infect the endophytic system of *A. americanum*. To further test this hypothesis, future research involving the use of isolates of *C. gloeosporioides* that have been genetically modified to express the green fluorescent protein of the jelly fish *Aequorea victoria* under the control of a constitutive promoter and in a controlled environment could be undertaken. Green fluorescent protein has revolutionized cellular biology (Heath, 2000; Lorang, 2001) and has been used to monitor gene expression (ie. Dumas et al., 1999) and the infection process (ie. Maor et al., 1998, Sexton and Howlett, 2001). An efficient transformation protocol for the transformation of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* has been reported (Robinson and Sharon, 1999) providing a method that can be utilized to transform an isolate of *C. gloeosporioides* collected from dwarf mistletoe. By utilizing an isolate of *C. gloeosporioides* that expresses green fluorescent protein under a controlled environment, time course experiments of the infection process can be conducted to follow hyphae in the infected shoot to unequivocally understand the extent of fungal colonization.

## Chapter 5: Incidence of *Colletotrichum gloeosporioides* on *Arceuthobium americanum* in the crown of lodgepole pine

### 5.1 Introduction

This study was conducted to observe the natural infection of *Arceuthobium americanum* by *Colletotrichum gloeosporioides* in the canopy of *A. americanum* infected *Pinus contorta* var. *latifolia*. The results of the survey may predict the efficacy of inundative biological control of *A. americanum* using *C. gloeosporioides* at all crown positions, based on the distribution of naturally infected *A. americanum*.

The interactions between the forest canopy and the atmosphere are very complex. Water vapour is released to the atmosphere through transpiration, and transpiration rates depend upon biological and structural attributes that influence local concentrations in the canopy, such as sunlight, temperature, turbulence, humidity and leaf physiological condition (Rose, 1996). It is expected that gradients of sunlight, relative humidity, wind speed and leaf wetness exist in the crown of lodgepole pine. The top and south facing portions of the crown are exposed to greater solar radiation and lower relative humidity than the lower and north facing portions of the crown. Similarly, the branch tip is exposed to greater solar radiation than the region of the branch located near the main stem which is more sheltered and likely have higher relative humidity than the branch tip.

Conidia of *C. gloeosporioides* are released from acervuli during rain and dew, and relative humidity is important during the infection process because conidia are rapidly inactivated by ultraviolet light and desiccation (Wastie, 1972). Free water is not required for conidia germination; however, germination is enhanced in the presence of free water (Wastie, 1972). Given the moisture requirements necessary for successful disease development by *C. gloeosporioides*, it is likely that a gradient of *C. gloeosporioides* infected *A. americanum* exists within the canopy of *A. americanum* infected lodgepole pine.

The hypothesis explored in this study is that successful establishment of *C. gloeosporioides* on *A. americanum* occurs on infections that are shaded and exposed to increased relative humidity. Three tests of this hypothesis were addressed in this study:

1. Infection of *A. americanum* by *C. gloeosporioides* is highest in the lower portion of

the crown, 2. Infection of *A. americanum* by *C. gloeosporioides* is highest in the north facing side of the crown, and 3. *A. americanum* infections located at the branch tip have lower *C. gloeosporioides* infection than sheltered *A. americanum* infections located near the main stem, all versus the null hypothesis of no significant difference.

## **5.2 Materials and Methods**

### **5.2.1 Stand and tree selection**

The stand selected for the study was located at 50° 29' 15"N latitude 121° 36' 19"W longitude and 1174m elevation in the Thompson dry cool variant of the interior Douglas-fir biogeoclimatic zone (Lloyd et al. 1990). The criteria for stand selection were: pure lodgepole pine, high dwarf mistletoe rating (DMR) (Hawksworth, 1977), *C. gloeosporioides* infection of the dwarf mistletoe present in the lower crown, mature trees with high live crown ratio and dwarf mistletoe infection throughout the crown. Six trees distributed throughout the selected stand were located and analyzed.

### **5.2.2 Data collection**

#### **5.2.2.1 Trees**

Diameter at breast height (DBH) was measured and the dwarf mistletoe rating was calculated based on Hawksworth's (1977) six-class system where 0 is no dwarf mistletoe infection and 6 is severe infection. The cardinal directions (aspect) were marked on the stem of each tree and the tree was then felled and the stem marked at 1-meter intervals. Tree height was recorded by measuring the length of the stem on the ground. A disk was cut from the stump for later aging of the tree.

#### **5.2.2.2 *Arceuthobium americanum***

Starting at the base of the tree, each branch was checked for the presence of *A. americanum*. If *A. americanum* was present on the branch, the height of the branch from the ground, aspect of the branch (quadrant centered on the cardinal direction), the distance of the *A. americanum* infection from the stem, total branch length, *A. americanum* sex, and the presence or absence of *C. gloeosporioides* on *A. americanum* shoots was recorded. *Colletotrichum gloeosporioides* was identified by the presence of

characteristic lesions on *A. americanum* shoots or fruit (Hawksworth and Weins, 1996). When systemic witches' brooms were encountered, they were treated as a single infection and all but infection location on the branch and branch length, were recorded.

### 5.2.3 Statistical Analysis

The vertical pattern of *C. gloeosporioides* infection was assessed by dividing the crown of each tree into thirds and calculating the percent infection of *A. americanum* by *C. gloeosporioides* in each third. In an alternative approach, the presence of *C. gloeosporioides* at different locations in the canopy was calculated by dividing the crown of the tallest tree into thirds of 0-5m, 6-10m and 11-16m. The location of each dwarf mistletoe infection on every tree was then assigned to one of these height classes. The aspect of *C. gloeosporioides* in the crown was analyzed by stratifying the data based on aspect and calculating the percent infection of *A. americanum* by *C. gloeosporioides*. Horizontal position within the crown was estimated by computing the ratio of infection location to total branch length.

Analysis of variance was used to compare percent infection of *A. americanum* by *C. gloeosporioides* at different crown and canopy heights and aspect positions. If the assumptions of normality and equal variance were not met, Kruskal-Wallis one-way analysis of variance on ranks was used; otherwise, two-way analysis of variance was used to compare the sampled trees and the treatment effect.

The Chi-square test of independence was used to analyze the relationship between the presence of *C. gloeosporioides* and *A. americanum* sex and the presence of *C. gloeosporioides* and *A. americanum* horizontal location on the branch. Horizontal location on the branch was a ratio between 0 and 1 and intervals of 0.1 were used to calculate the chi-square statistic. All statistical analyses were computed using the computer package SigmaStat 2.03 (SPSS Inc, Chicago, IL).

## 5.3 Results

### 5.3.1 Individual trees

Dwarf mistletoe infection of the host tree was highly variable. All trees were heavily infected, having an average DMR of 5.5 (Table 5-1), but the actual number of

dwarf mistletoe infections ranged from 28 to 323, averaging  $126 \pm 44$  (mean  $\pm$  standard error of the mean) (Table 5-1). The range of dwarf mistletoe infection of the host tree was broad; however, infection of *A. americanum* by *C. gloeosporioides* was very consistent, averaging  $23.0 \pm 1.1$  % (Table 5-1).

Table 5-1. Characteristics of individual trees surveyed in this study.

Tree	Height (m)	DBH (cm)	Age (years)	DMR	# DM infections	# DM with <i>Colletotrichum</i>	% infection
1	12.6	20.4	117	6	127	26	20.5
2	8.6	17.4	103	5	28	6	21.4
3	15.4	21.5	104	5	156	36	23.1
4	9.7	18.5	138	6	72	17	23.6
5	14.5	19.6	75	5	50	14	28.0
6	12.3	18.6	104	6	323	68	21.1
Mean	12.2	19.3	107	5.5	126	28	23.0
SE <sup>†</sup>	1.1	0.6	8.4	0.2	44	9	1.1

<sup>†</sup> Standard error of the mean

### 5.3.2 Crown position

The presence of *C. gloeosporioides* infected *A. americanum* at different vertical locations within the host tree crown is shown in table 5-2. Kruskal-Wallis one-way ANOVA on ranks was used to analyze this data because the assumption of normality was not met. There was no significant difference in the presence of *C. gloeosporioides* between the trees ( $P=0.835$ ); therefore, the crown thirds were pooled and crown position was compared. Kruskal-Wallis one-way ANOVA on ranks indicated that there were no significant differences between crown position and the presence of *C. gloeosporioides* ( $P=0.194$ ). These results are illustrated graphically in figure 5-1.

Table 5-2. Infection of *A. americanum* by *C. gloeosporioides* at different tree crown thirds.

Tree	Bottom Third			Middle Third			Top Third			All
	# DM	# Inf	% Inf	# DM	# Inf	% Inf	# DM	# Inf	% Inf	
1	5	1	20.0	42	11	26.2	80	14	17.5	20.5
2	1	1	100	7	5	71.4	20	0	0	21.4
3	46	4	8.7	80	27	33.8	30	5	16.7	23.1
4	24	4	16.7	38	9	23.7	10	4	40	23.6
5	10	2	20.0	24	7	29.2	16	5	31.3	28.0
6	127	23	18.1	180	41	22.8	16	4	25.0	21.0
Mean			30.6			34.5			21.8	22.9

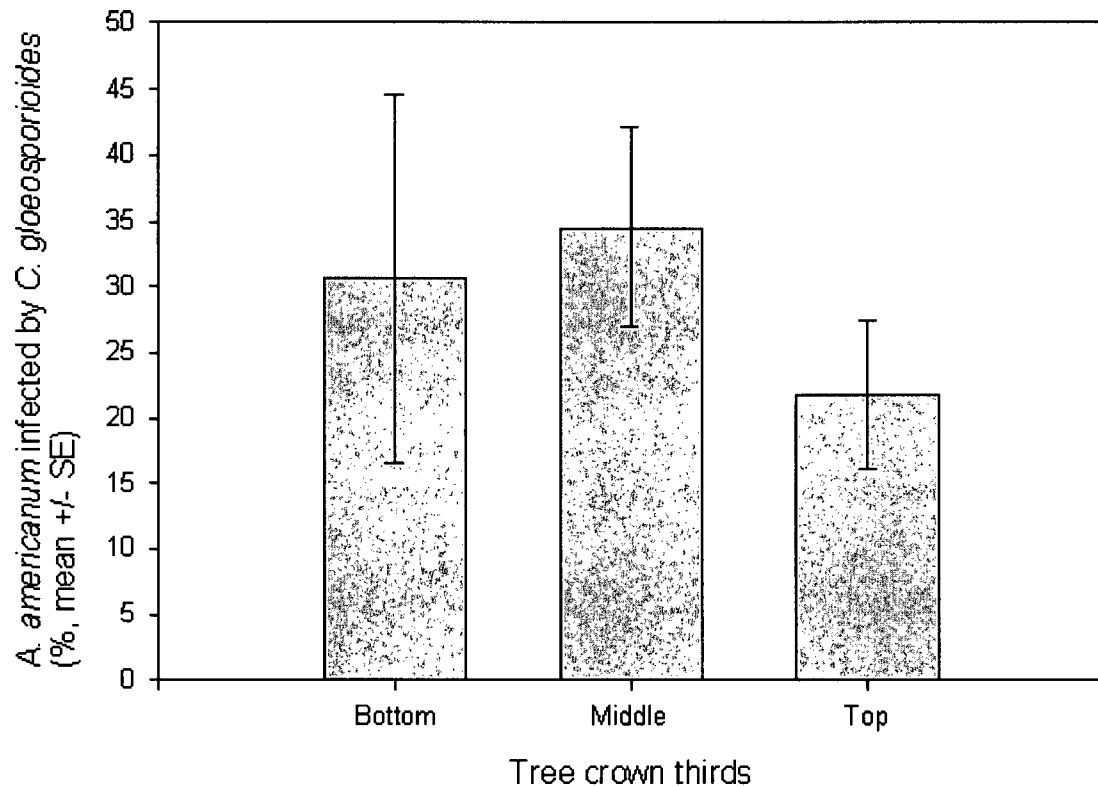


Figure 5-1. Percent infection of *A. americanum* by *C. gloeosporioides* in different tree crown thirds. Mean  $\pm$  standard error of the mean.

### 5.3.3 Canopy position

The canopy of the stand was divided into thirds based on the height of the tallest tree sampled and the *A. americanum* infections assigned to thirds by their height above ground. Percent infection of *A. americanum* by *C. gloeosporioides* at different stand canopy positions was analyzed by two-way ANOVA because the assumptions of normality and equal variance were met. The two-way ANOVA results indicated that there was no significant difference in infection by *C. gloeosporioides* between trees ( $P=0.939$ ) or canopy position ( $P=0.423$ ) (Appendix 4). Canopy position data was pooled and is shown graphically in figure 5-2. Figure 5-3 illustrates graphically the distribution of *A. americanum* within the canopy. As can be seen in figure 5-3, 91% of the total *A. americanum* in the sampled trees was located in the bottom two-thirds of the canopy and *C. gloeosporioides* was present in all canopy positions.

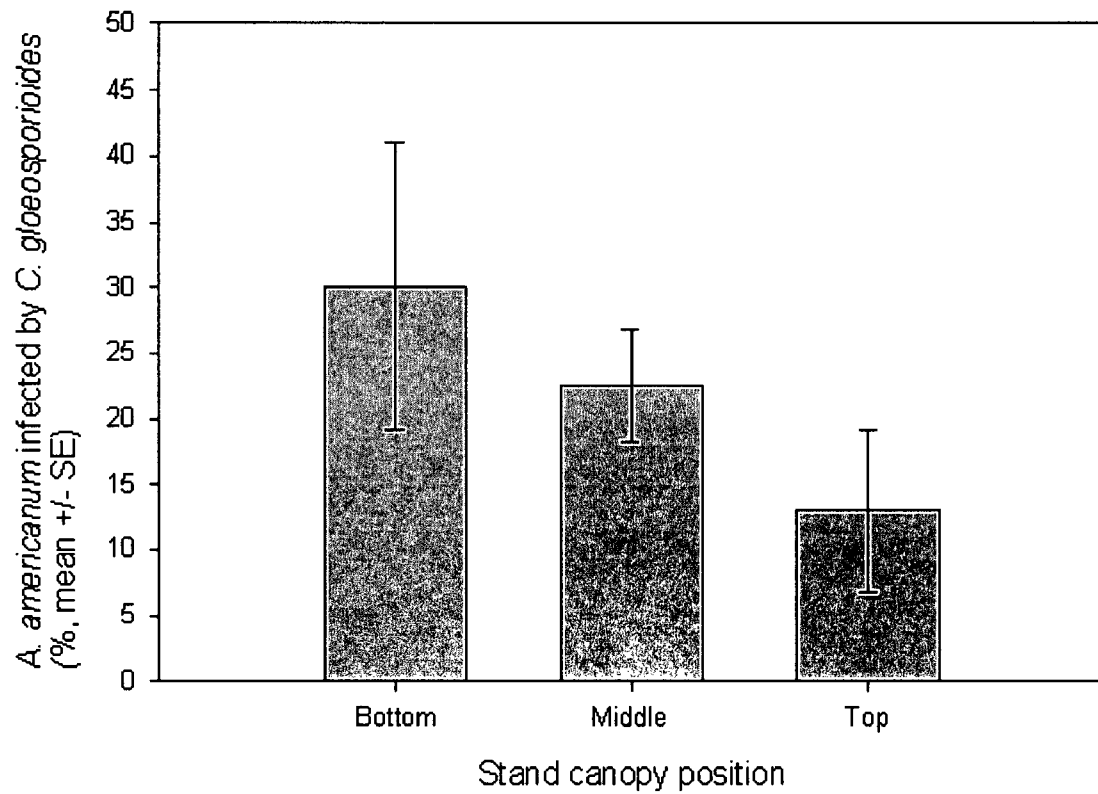


Figure 5-2. Percent infection of *A. americanum* by *C. gloeosporioides* at different locations in the stand canopy. Mean  $\pm$  standard error of the mean.

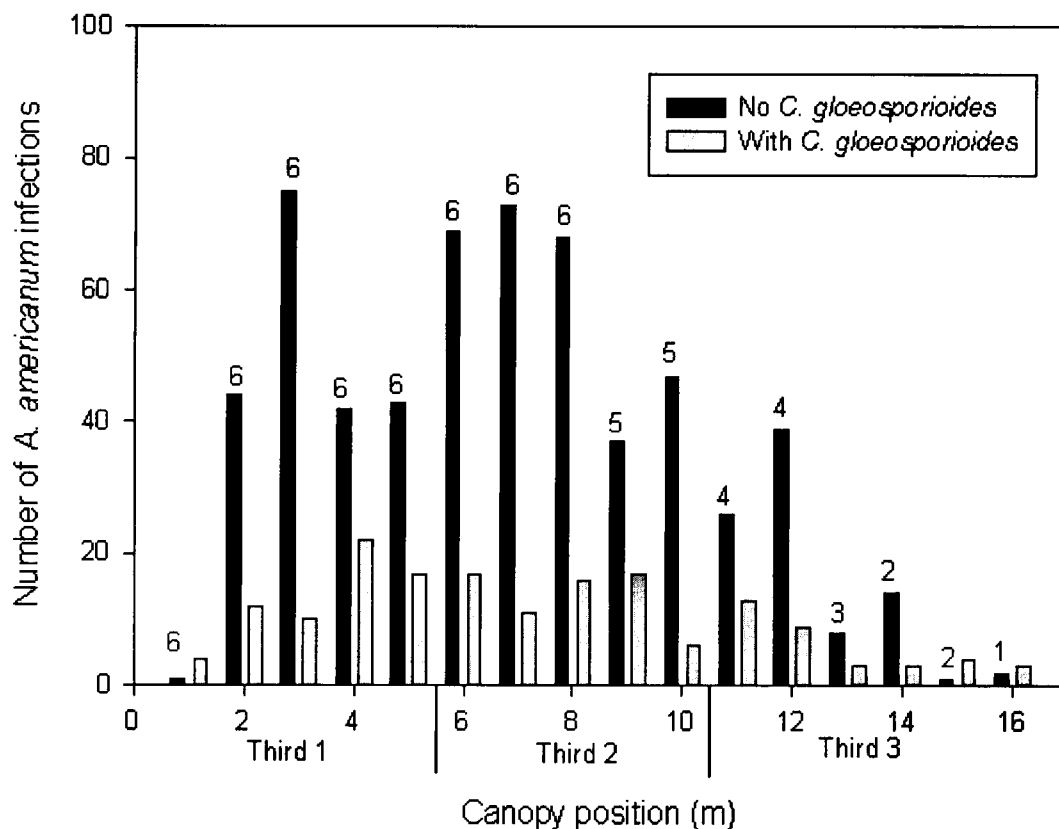


Figure 5-3. Distribution of *A. americanum* within the canopy. Numbers above bars represent the number of trees having crowns within the interval.

#### 5.3.4 Canopy aspect

Two-way ANOVA indicated no significant difference between percent *A. americanum* infection by *C. gloeosporioides* between trees ( $P=0.591$ ) or aspect ( $P=0.914$ ) (Appendix 4). Percent infection of *A. americanum* at different aspect locations was pooled and is shown graphically in figure 5-4.

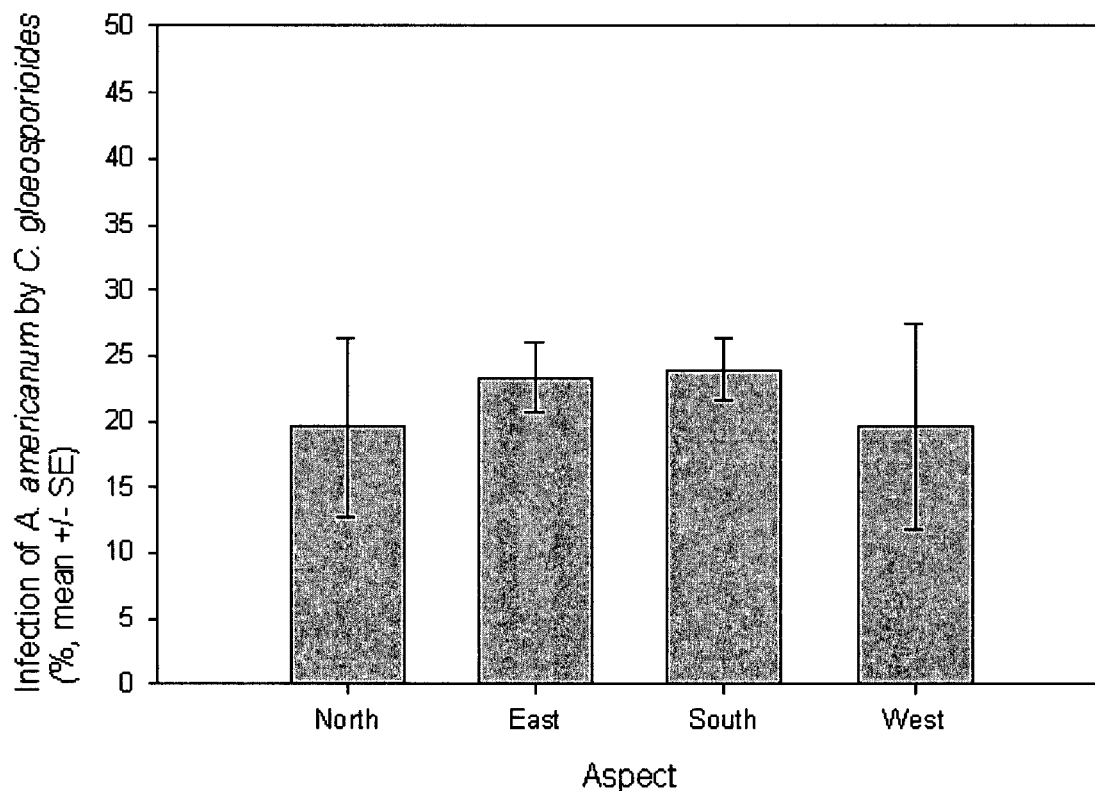


Figure 5-4. Percent infection of *A. americanum* by *C. gloeosporioides* at different aspects. Mean  $\pm$  standard error of the mean

#### 5.3.5 *A. americanum* sex and the presence of *C. gloeosporioides*

The relationship between the presence of *C. gloeosporioides* and sex was analyzed using the chi-square test of independence and it was found that sex and the presence of *C. gloeosporioides* did not depend upon *A. americanum* sex ( $\chi^2$  with 1 degree of freedom = 0.578;  $P=0.447$ ).

#### 5.3.6 *A. americanum* horizontal canopy position

The location of *A. americanum* infection on the infected branch was calculated using the ratio of infection location over branch length. The frequency distribution of infected and uninfected *A. americanum* at different branch positions is presented in figure 5-5. Calculation of the chi-square statistic based on the intervals 0-0.6, 0.61-0.8, 0.81-

0.9, and 0.91-1.0 indicated that the presence of *C. gloeosporioides* could not be shown to be dependent upon horizontal position on the branch ( $\chi^2$  with 3 degrees of freedom = 2.484;  $P=0.478$ ).

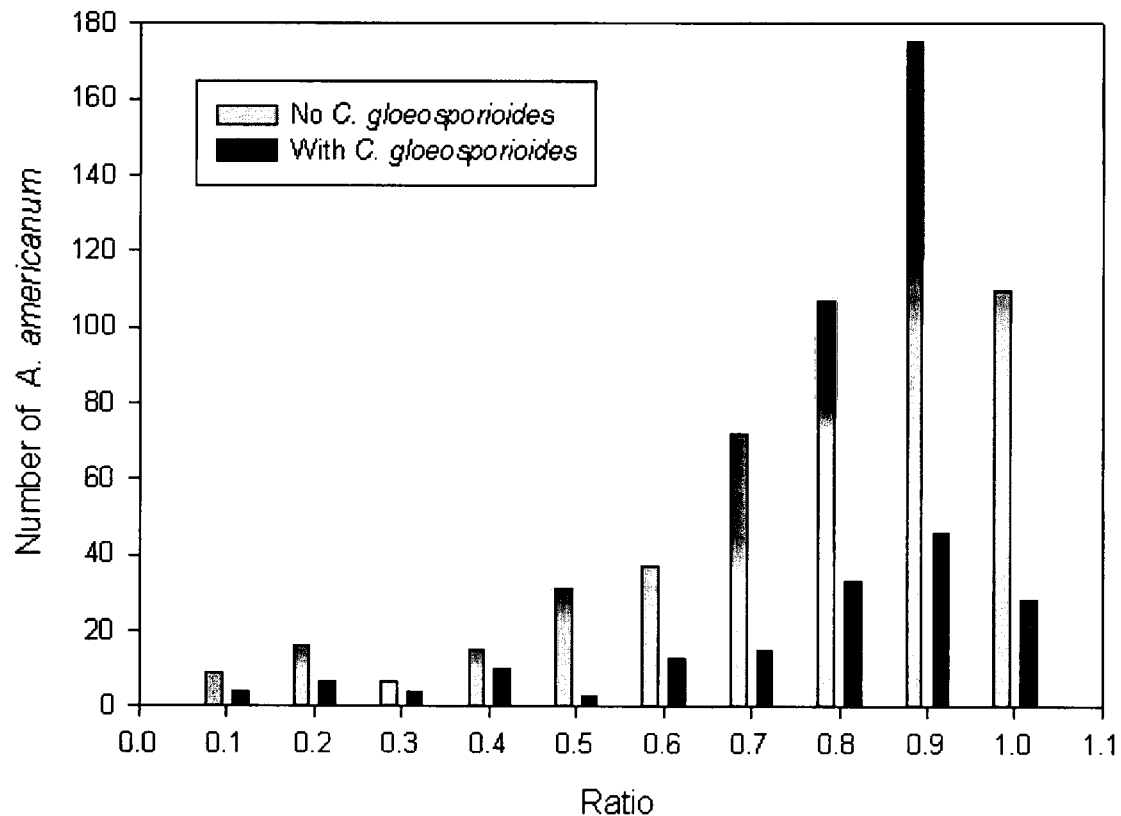


Figure 5-5. Distribution of *C. gloeosporioides* infected and uninfected *A. americanum* on the host branch. Values close to 0 represent infections close to the main stem; values close to 1 represent infections close to the branch tip.

#### 5.4 Discussion

The survey described here was conducted to observe the natural distribution of *C. gloeosporioides* infection of *A. americanum* within the crown of *Pinus contorta* var. *latifolia*. The distribution of *C. gloeosporioides* within the canopy was assessed to determine if there were any factors, such as solar radiation, that would limit the efficacy of an inundative biological control agent. The survey was relatively limited in scope, as only six trees within one stand were surveyed and it is possible that the results observed

here would not be observed in a stand with different structural characteristics given the complex relationships between the canopy and the atmosphere.

The occurrence of *C. gloeosporioides* infection of dwarf mistletoe has been surveyed on *Arceuthobium abietinum* Englm. Ex Munz f. sp. *magnificae* Hawksworth and Weins (syn. *Arceuthobium campylopodum* Engelm. f. *abietinum* (Englem.) Gill) (Parmeter et al., 1959), *Arceuthobium campylopodum* Englm in Gray (Wicker, 1967) and *Arceuthobium americanum* (Muir, 1977). The percent infection of dwarf mistletoe by *C. gloeosporioides* observed in this study was lower than previously reported, averaging 22.9%. Muir (1977) observed 75% infection of plants at one site and 35.5% infection at another site. Wicker (1967) observed *C. gloeosporioides* infection of 67% of *A. campylopodum* on 75% of *P. ponderosa* trees sampled, while 100% of *P. contorta* var. *latifolia* trees surveyed in this study were parasitized by *A. americanum* that was infected by *C. gloeosporioides*. Parmeter (1959) observed 27.7% *C. gloeosporioides* infection of *A. abietinum* f. sp. *magnificae* plants. The variation in percent infection may relate to the ability of the fungus to infect different *Arceuthobium* hosts, alternatively, it could be due to different environmental conditions that affect inoculum and symptom production at the time of sampling or environmental differences between locations that affect *C. gloeosporioides* disease development.

The frequency of *C. gloeosporioides* was not significantly different between trees, crown thirds or canopy thirds; compared to the vertical distribution described by Parmeter et al (1959) who observed uniform infection of the dwarf mistletoe in the upper  $\frac{3}{4}$  of the crown and a decrease in the lower  $\frac{1}{4}$  of the crown. The observation of no significant difference between crown or canopy thirds suggests that no gradients of relative humidity or solar radiation exist in the canopy of the stand that was sampled, or that if gradients were formed, they did not limit *C. gloeosporioides* infection. The stand that was sampled had been spaced and pruned from 1667 stems per hectare to 1208 stems per hectare 6 years prior to this study, thus the stand was relatively open. This stand was selected because the trees had a high live crown ratio, which was a necessary component of the study. It was desirable to sample trees that had foliage at all canopy positions because understory trees or overstory trees do not represent all canopy positions. The openness of the stand selected however may have prevented the formation of a relative

humidity gradient within the crown because the lower crown was not sheltered from the wind, as would be the case if stand density were higher. Light penetration to the lower portion of the crown was also increased. The environmental conditions at the different canopy positions are likely to be representative of the conditions present in the upper canopy of a dense stand. The observation of *C. gloeosporioides* infection in the top third of the canopy is important from a biological control standpoint because it indicates that the *A. americanum* infections in the upper crown, which are the most important for *A. americanum* spread, are as susceptible to *C. gloeosporioides* infection as *A. americanum* in the lower crown. This finding also suggests that the results of the field trial of chapter 2 may also be applicable to upper crown *A. americanum* infections and open stands. In the future, if it is desirable to create a model to describe the infection of *A. americanum* by *C. gloeosporioides*, the model may be based on either crown or canopy position, as the trend was the same in both analyses.

Aspect of *C. gloeosporioides* infected *A. americanum* and branch location were not related to infection of *A. americanum* by *C. gloeosporioides*. This suggests that solar radiation did not affect disease development. Shading of the crown by other trees in the stand may have decreased the amplitude of the gradient. The effect of aspect may also be reduced by the fact that the south facing side of one tree and the north facing side of an adjacent tree occupy the same space and may be exposed to very similar conditions. An alternative explanation for the lack of a gradient of *C. gloeosporioides* infection of *A. americanum* is that *C. gloeosporioides* establishment likely occurs on cloudy days with high relative humidity, in which case, strong gradients of light do not exist.

Dwarf mistletoe sex and the presence of *C. gloeosporioides* were independent, suggesting that pistillate and staminate *A. americanum* plants are equally susceptible to infection by *C. gloeosporioides*, as was observed by Wicker (1967). This is not surprising as *C. gloeosporioides* does not specifically parasitize one *A. americanum* tissue type, unlike *Caliciopsis arceuthobii*, which only parasitizes female flowers (Kuijt, 1969).

This survey was conducted to observe any trends present in natural infection of *A. americanum* by *C. gloeosporioides* that might suggest reduced efficacy of an inundative biological control agent in certain crown locations. All tests failed to reject the null

hypothesis; results of this survey indicated that there were no significant differences between crown and canopy thirds, aspect within the crown or branch position and infection of *A. americanum* by *C. gloeosporioides*. This suggests that fungal inoculum is not limiting at different crown positions, or that it is equally limited at all positions, and environmental conditions were suitable for disease development at all crown positions. These results imply that fungal inoculum applied during biological control treatment, applied under appropriate climatic conditions, has an equal probability of causing disease on *A. americanum*, regardless of crown position.

## Chapter 6 – Infection of *Arceuthobium americanum* by *Caliciopsis arceuthobii*

### 6.1 Introduction

*Caliciopsis arceuthobii* (ex. *Wallrothiella arceuthobii*) is an ascomycete fungus that parasitizes the female flowers of the spring flowering dwarf mistletoes *Arceuthobium americanum*, *A. douglasii*, *A. pusillum*, and *A. vaginatum* subsp. *cryptopodium* (Hawksworth and Wiens, 1996). Ascospores mature in perithecia in March and April and are liberated during flowering. It is hypothesized that ascospores are then transported to other flowers by insects, where they germinate and infect the female flower, preventing fruit development; therefore, pollination and disease initiation occur simultaneously. Perithecia are visible in the fall of the year of infection, but asci and ascospore development is delayed until the following spring, resulting in a life cycle that requires one year (Kuijt, 1969). Weir (1915) suggested using *C. arceuthobii* as a biological control agent and was able to infect *A. americanum* by binding diseased *A. douglasii* to a branch infected by *A. americanum*. Utilization of *C. arceuthobii* as a biological control agent for *A. douglasii* was investigated and inoculation with crushed perithecia resulted in 17% infection of 318 fruits (Knutson and Hutchins, 1979).

The effect of *C. arceuthobii* on *A. americanum* under natural conditions was investigated in this study. A population of *C. arceuthobii* infected *A. americanum* was located and the percent fruit reduction caused by *C. arceuthobii* and the spread of *C. arceuthobii* to healthy *A. americanum* was quantified. The effect of the fungus on fruit production by *A. americanum* and the potential of this fungus as a biological control agent for *A. americanum* are discussed.

### 6.2 Materials and Methods

#### 6.2.1 Stand characteristics

The stand selected for this study was located at 52° 02' 54"N, 121° 48' 45"W, 1023 meters, within the Knife Creek Block of the Alex Fraser Research Forest, near 150 Mile House, British Columbia. The site was classified as the dk3 variant of Interior Douglas-Fir zone (IDFdk3) under the biogeoclimatic ecosystem classification system

(Steen and Coupé, 1997). Individual female *A. americanum* swellings were located on lodgepole pine trees that averaged 7.4 cm diameter at breast height and approximately 8 m in height. Prior to spacing to 1500 stems per hectare in 1990, the stand was assessed at 4699 coniferous stems per hectare (93% lodgepole pine, 3% Douglas-fir and 2% interior spruce). This stand was selected because *C. arceuthobii* was present and because it was located within the University of British Columbia research forest.

### 6.2.2 *Arceuthobium americanum* selection

In 1998, 30 female *A. americanum* infections were randomly selected and tagged. *Caliciopsis arceuthobii* was present on the flowers of 22 of the 30 selected infections. Every female *A. americanum* infection selected bore a crop of fruit when the trial was initiated. *Arceuthobium americanum* infections were located on the branches and stems of the host trees and all infections observed were in the lower crown.

### 6.2.3 Data collection and analysis

The initial data were recorded May 15, 1998 and assessment occurred July 28, 1999 and August 17 of 2000 and 2001. At every assessment, the number of fruit and number of flowers bearing *C. arceuthobii* perithecia were counted. During the 2001 assessment, the number of healthy fertilized flowers was counted to estimate fruit production in 2002.

The percent reduction in fruit production caused by *C. arceuthobii* infection in the first year of the study was calculated assuming that the fruit present in May of 1998 were produced on flowers that escaped *C. arceuthobii* infection in 1997 and that the perithecia observed were on the same cohort of flowers that were producing fruit in 1998. The percent reduction in fruit production was therefore calculated according to the formula:

$$\% = \left[ \frac{\alpha}{(\alpha + \beta)} \right] \times 100$$

where  $\alpha$  = number of flowers with perithecia in May 1998,  $\beta$  = number of fruit present in May 1998.

The percent reduction in fruit production in 1999 could not be calculated because the 1998 cohort of *C. arceuthobii* perithecia was not recorded<sup>1</sup>. The percent fruit reduction for 2000 and 2001 was quantified assuming that fruit present in year x are a result of flowers that escaped infection in year x-1 and that *C. arceuthobii* observed in year x will cause a reduction in fruit production in year x+1 using the formula:

$$\% = \left[ \frac{\alpha}{(\alpha + \chi)} \right] \times 100$$

where  $\alpha$  = number of flowers with perithecia in year x-1,  $\chi$  = number of fruit present at year x.

The percent reduction in fruit production for 2002 was predicted based on the number of *C. arceuthobii* infected flowers and the number of *C. arceuthobii* free flowers using the formula:

$$\% = \left[ \frac{\alpha}{(\alpha + \delta)} \right] \times 100$$

where  $\alpha$  = number of flowers with perithecia in 2001,  $\delta$  = number of flowers that escaped *C. arceuthobii* infection in 2001.

To clarify the relationship between perithecia production by *C. arceuthobii* and fruit production on *A. americanum*, lifecycle drawings are presented in Appendix 5.

To test the hypothesis that *C. arceuthobii* infected *A. americanum* is significantly more likely to have *C. arceuthobii* infection in the following year than *A. americanum* without *C. arceuthobii*, versus the null hypothesis of random infection, the Fisher exact test was calculated using SigmaStat 2.03. Two by two contingency tables for 1999 and

---

<sup>1</sup> The road to the field site was closed due to heavy rain when I attempted to record data from June 25-27, 1999; therefore, the 1998 cohort of *C. arceuthobii* perithecia was not assessed.

2000, and 2000 and 2001 were constructed with infected in year  $x$  and not infected in year  $x$  versus infected in year  $x+1$  and not infected in year  $x+1$ . Only *A. americanum* infections that were alive in both years and had shoots in both years were included in the analysis. Only the years 1999 vs. 2000 and 2000 vs. 2001 were plotted because the *C. arceuthobii* perithecia observed in 1998 represent the 1997 cohort. Due to the timing of field data collection, the 1998 cohort of *C. arceuthobii* perithecia were not recorded (Appendix 5).

## **6.3 Results**

### **6.3.1 *Caliciopsis arceuthobii* infection of *Arceuthobium americanum***

Infection of *A. americanum* by *C. arceuthobii* was obvious in August of the year of infection. In Figure 6-1, taken in August 2001, fruit, *C. arceuthobii* infected and uninfected flowers of *A. americanum* are visible. Arrows 1 and 2 of Figure 6-1 point to *C. arceuthobii* infected and uninfected flowers, respectively, that were produced in 2001, while arrow 3 points to a fruit that has developed as a result of escaping *C. arceuthobii* infection in 2000. Arrow 4 of Figure 6-1 points to an *A. americanum* flower that was infected by *C. arceuthobii* in 2000 and is dying.

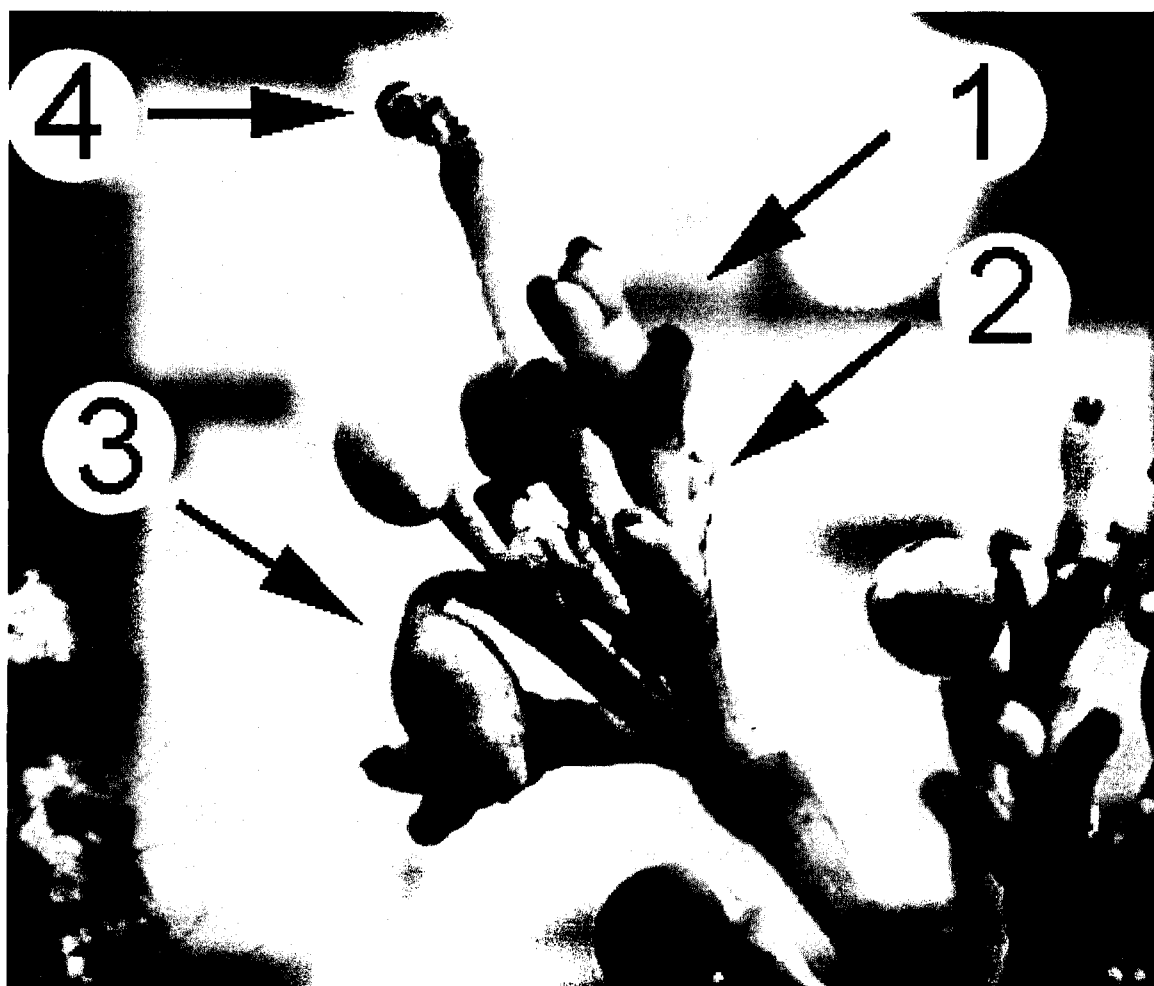


Figure 6-1. *Arceuthobium americanum* infected by *Caliciopsis arceuthobii*. Photo taken August 17, 2001 at the Knife Creek block of the Alex Fraser Research Forest, near 150 Mile House, British Columbia. 1. *Caliciopsis arceuthobii* perithecia present on *A. americanum* flower infected in 2001. 2. *Arceuthobium americanum* flower that escaped infection in 2001. 3. Maturing fruit of *A. americanum* that escaped infection in 2000. 4. *Arceuthobium americanum* flower that was infected by *C. arceuthobii* in 2000.

### 6.3.2 Fruit production on *A. americanum*

The average percent fruit reduction caused by *C. arceuthobii* was found to range from a low of 46% in 1998, to a high of 72% in 2000. The predicted percent fruit reduction caused by *C. arceuthobii* in 2002 is 39% (Table 6-1). Variation in the amount

of fruit reduction on *C. arceuthobii* infected *A. americanum* was high, ranging from 1% to 100%. The average fruit reduction per year, caused by *C. arceuthobii*, over the course of the experiment, not including the predicted fruit reduction, was 58%.

Table 6-1. Average reduction in fruit production caused by *Caliciopsis arceuthobii* infection of *Arceuthobium americanum*.

Year	Average % reduction	Standard error of the mean
1998	46	7.4
1999	Cannot calculate	
2000	72	10.8
2001	53	13.1
2002 <sup>†</sup>	39	11.0

<sup>†</sup>Predicted

Fruit production on individual *A. americanum* infections was found to be variable throughout the course of the experiment; large crops of fruit were not sustained on single infections over the course of the experiment and different infections had maximum fruit production in different years. Fruit production averaged 58, 23, 11 and 20 fruit per *A. americanum* infection in 1998, 1999, 2000 and 2001 respectively.

When the number of *C. arceuthobii* infected flowers at year x was plotted against the number of *C. arceuthobii* infected flowers at year x + 1 (Figure 6-2), no relationship was observed and there was no significant linear regression ( $P=0.271$ ). This finding implies that the inoculum load present in year x does not affect infection of *A. americanum* the following year.

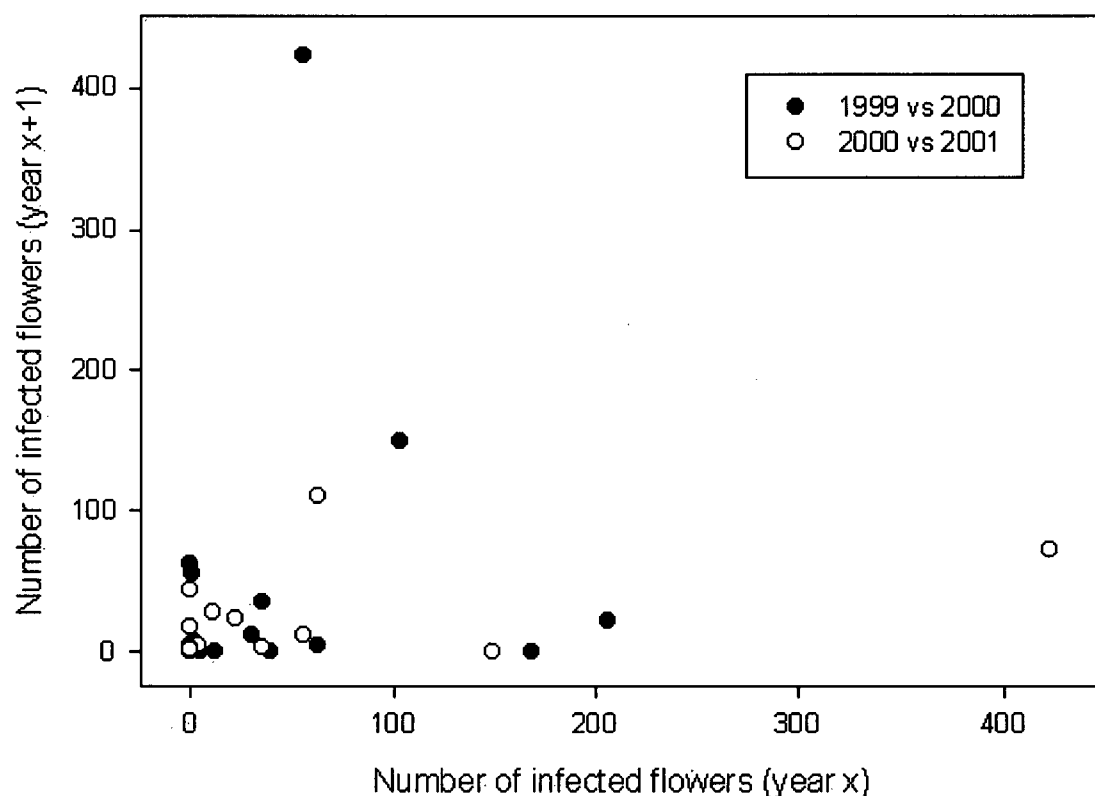


Figure 6-2. Scatter to compare the number of *C. arceuthobii* infected *A. americanum* flowers at year  $x$  with the number of infected flowers at year  $x+1$ . In the 1999 vs 2000 plot, 6 data points are located at the origin, while in the plot of 2000 vs 2001, 2 data points are at the origin.

### 6.3.3 Movement in the stand

Using the Fisher exact test to test the hypothesis that having *C. arceuthobii* in one year significantly predisposed the *A. americanum* infection to having *C. arceuthobii* in the following year failed to reject the null hypothesis for both pairs of successive years. Infection of *A. americanum* by *C. arceuthobii* was not significantly different than expected from random occurrence.

Initially, eight of the *A. americanum* infections selected in this experiment were not infected by *C. arceuthobii*. One year later one replicate was dead and two were infected by *C. arceuthobii*. Of the two replicates that became diseased, one showed no sign of disease in 2000 or 2001 and one had perithecia in 1999 and 2000 and was dead in

2001. One replicate had no sign of *C. arceuthobii* in 1998 or 1999 and was dead in 2000 and one replicate had no sign of *C. arceuthobii* in 1998, 1999, or 2000 and was dead in 2001. One replicate that had no *C. arceuthobii* in 1998, 1999, or 2000 bore perithecia in 2001. Two of the replicates remained free of *C. arceuthobii* throughout the experiment.

Of the 22 replicates that had perithecia at the start of the experiment, six were dead in 1999. Six replicates had perithecia present every year for four years, while one replicate bore perithecia in 1998 only. Four replicates had *C. arceuthobii* perithecia in 1998 and 2001 only, three replicates had perithecia in 1998, 1999 and 2001, one replicate had perithecia in 1998, 1999 and 2000, and one replicate had perithecia in 1998, 2000 and 2001. The raw data are presented in Appendix 6.

#### 6.4 Discussion

From a biological control perspective, the survey suggests that *C. arceuthobii* can reduce the spread and intensification of *A. americanum* by a significant amount through interfering with the production of new seed. Although only one stand was selected, the mode of action of this hyperparasite suggests that wherever it is present, it will cause a reduction in seed production. One of the attributes of a biological control agent for dwarf mistletoe, as defined by Wicker and Shaw (1968), is that the biological control agent must have an efficient mode of restricting development of the target disease. Clearly, *C. arceuthobii* meets this requirement by infecting the female flowers. Following the development of *C. arceuthobii* within the population of *A. americanum* surveyed indicated that it persists over many years on the host, affecting many seed crops, and that it can readily move from diseased individuals to healthy individuals. Although these traits are not specifically outlined by Wicker and Shaw (1968), they are important components of a biological control strategy. Spread to *A. americanum* infections that escaped initial biological control application is likely to occur as *A. americanum* that was *C. arceuthobii* free in one year was occasionally observed to be infected by *C. arceuthobii* in the following year. For *C. arceuthobii* to be maintained in the stand, *A. americanum* must also be maintained, and as the density of *A. americanum* increases, it is expected that disease caused by *C. arceuthobii* will also increase.

Infection of *A. americanum* by *C. arceuthobii* in one year was not related to the presence of *C. arceuthobii* in the preceding year. This suggests that if insects vector the fungus, as suggested by Kuijt (1969), insects travel readily between infected and healthy *A. americanum* flowers and that the ascospores remain viable for a relatively long period of time. If the fungus is dispersed by splash dispersal or wind, it would be expected that *A. americanum* infections with many perithecia would have a high infection rate in the following year, however, the scatter plot in figure 6-2 does not support this.

Although pathogenesis process of *C. arceuthobii* results in a natural level of control of *A. americanum*, some of the features of the *Caliciopsis* / *Arceuthobium* / lodgepole pine pathosystem described in Chapter 2 suggest that *Caliciopsis* may not be a good candidate for a biological control agent. The range of *C. arceuthobii* was the same as *C. gloeosporioides*, but *C. arceuthobii* was observed in 8 sites and *C. gloeosporioides* was isolated from 21. When perithecia were collected from *A. americanum*, *C. arceuthobii* was not successfully isolated, proving that it is difficult to culture. *Caliciopsis arceuthobii* has been cultured, but it grows slowly (Parker, 1970; Knutson and Hutchins, 1979), which is a possible reason why it was not isolated during the work conducted for this thesis. *Cladosporium* sp. is commonly associated with the perithecia of *C. arceuthobii* (Kuijt, 1969) and that fungus was commonly isolated in the survey portion of Chapter 2. The difficulty of culturing *C. arceuthobii*, the slow growth in culture, and the fact that no perithecia, and thus ascospores, have been produced in culture (Knutson and Hutchins, 1979), suggest that inoculum production necessary for biological control application would be difficult. The asexual stage of *C. arceuthobii* is unknown, but it is possible that if an asexual stage exists, conidia production in culture could provide inoculum for biological control studies. Inoculation studies that have been carried out with *C. arceuthobii* have used crushed perithecia in water (Knutson and Hutchins, 1979) and diseased *A. douglasii* placed in close proximity to *A. americanum* (Weir, 1915), but neither of these is practical at a commercial scale. Timing of application in the field must be synchronized with the flowering period of *A. americanum* to result in successful establishment. The stigmata of *A. americanum* is infected by *C. arceuthobii*, which is hypothesized to be inoculated by an insect vector. This may be difficult to replicate during biological control treatment application.

*Caliciopsis arceuthobii* has several characteristics that suggest that it would be an excellent biological control agent for *Arceuthobium americanum*. Unfortunately, there are several technical difficulties with culturing and inoculum preparation, which are major constraints in the development of inundative biological control agents (Auld and Morin, 1995), that must be overcome before this fungus can be utilized as a biological control agent for *Arceuthobium americanum*.

## Chapter 7 – *Arceuthobium americanum* response to shoot removal

### 7.1 Introduction

Biological control of dwarf mistletoes differs from biological control of weeds because death of the aerial shoots may not ultimately result in death of the dwarf mistletoe infection. The endophytic system within the host branch can survive to produce a new crop of shoots after the shoots are killed (Hawksworth, 1972). If a biological control agent were able to infect the endophytic system and prevent the production of new shoots, re-application of the biological control agent would not be required, unless secondary inoculum production caused a reduction in new shoot production. If however, the biological control agent does not infect the endophytic system, the production of new shoots and new fruit is likely to occur. The production of a new crop of seeds after biological control application would necessitate reapplication of the biological control agent prior to the new seeds being cast if the canopy of the trees being protected has not grown above the treated dwarf mistletoe infections. The most efficient timing of biological control treatment would be during the spring, one year after pollination of the female flowers, immediately prior to fruit dispersal when environmental conditions for *C. gloeosporioides* establishment are favourable. This scenario is similar to the effect of Ethephon, an ethylene releasing compound that causes rapid dwarf mistletoe shoot abscission but does not affect the endophytic system, resulting in renewed fruit production following treatment (Nicholls, 1988).

Application of an inundative biological control agent to dwarf mistletoe infections may result in uneven distribution of disease on the aerial shoots, allowing some shoots to escape infection and continue fruit production. The result of this scenario is dwarf mistletoe infections that have new shoots developing at the same time that fruit are maturing on shoots that escaped inoculation. It is not known if the dwarf mistletoe responds to partial shoot removal by increasing the production of new shoots or by increasing fruit production on existing shoots or if it modifies growth characteristics at all.

The first objective of this research was to observe how *Arceuthobium americanum* responds to biological control through mimicking the effect of a biological

control agent using manual shoot removal. The specific hypotheses examined were: 1) Shoot removal induces change in the growth rate of the endophytic system of *A. americanum*, 2) As damage to the aerial system of *A. americanum* increases, bud (=new shoot) production increases, 3) As damage to the aerial system of *A. americanum* increases, fruit production increases on the remaining shoots, 4) When the top portion of the shoot is damaged, *A. americanum* responds by increased fruit production on the existing shoots rather than increasing new shoot production, and 5) the average number of shoots, maximum shoot length and number of fruit remains constant over years when *A. americanum* is not damaged.

The second objective of this research was to estimate the treatment periodicity necessary to prevent *A. americanum* seed production through mimicking the action of a biological control agent that was assumed to remove all of the aerial shoots but had no effect on the endophytic system of the dwarf mistletoe.

## **7.2 Materials and Methods**

To investigate the effects of partial *A. americanum* infection and the effects of killing the aerial system, but not the endophytic system of *A. americanum*, field trials were established that mimicked the action of the biological control agent by manually removing shoots.

### 7.2.1 Partial shoot removal experiment.

The partial shoot removal experiment was designed to observe how the dwarf mistletoe plant responded following manual shoot removal that mimicked uneven establishment of a biological control agent. The experiment was established near Lytton (50° 27.324' N 121° 35.704' W 1166 m), British Columbia in the dry cool variant of the Interior Douglas-Fir zone (IDF dk1). Dwarf mistletoe infections that were selected were healthy individual *A. americanum* infections that were located on lodgepole pine trees approximately 30 years of age, averaging 7 cm diameter at breast height and 8 m in height. The overstory was composed of *A. americanum* infected lodgepole pine 80 – 100 years of age. Data collected prior to treatment included: *A. americanum* swelling diameter (measured at the point of maximum diameter), *A. americanum* swelling length, maximum shoot length, number of shoots and number of fruit (female). *Arceuthobium americanum* swelling diameter, swelling length and maximum shoot height were measured using digital calipers. The rate of growth of the endophytic system of *A. americanum* cannot be measured directly; therefore, in these experiments, the rate of *A. americanum* swelling diameter and length change was utilized as an index of endophytic system growth.

The experiment was initiated in July 1999, prior to seed dispersal and assessed prior to seed dispersal in August one and two years after treatment application. One and two years following treatment, the number of buds (defined as shoots <5 mm in length) was counted and at 2 years the number of pollinated female flowers was counted as a predictor of fruit production three years after treatment. Pollinated female flowers could be distinguished from unpollinated flowers based on size. The treatments and number of replicates for each sex are outlined in table 7-1. The number of shoots was counted prior to treatment and then the designated percent reduction in shoot number was accomplished by breaking the shoots off at the basal cup. When treatment 4 was applied, scissors were used to trim each shoot at the midpoint. The number of fruit present immediately following treatment was not counted. The number of female replicates was variable because 7 infections that were assumed to be male due to the lack of fruit were actually female infections that were not yet producing fruit. These female infections were added to the female replicates and more male infections were located and treated to bring the

number of male replicates to 10 per treatment. When the maximum shoot length was measured after treatment application, the maximum bud length was utilized for the maximum shoot length of the replicate if no shoots were present because buds are immature shoots.

Table 7-1. Treatments applied at the partial shoot experiment and number of replicates.

Treatment	Description	Male (n=)	Female (n=)
1	25% of aerial shoots removed.	10	11
2	50% of aerial shoots removed.	10	11
3	75% of aerial shoots removed.	10	14
4	All shoots trimmed to half original height.	10	11
5	No shoots removed.	10	10
6	All shoots removed.	10	10

### 7.2.2 Statistical analysis

Analysis of variance (ANOVA) was used if the assumptions of a normal distribution and equal variance were met. If either assumption was not met, analysis of variance on ranks was used to detect differences due to treatment one and two years after treatment. If differences were detected, Tukey's test or Dunn's test was used to determine which treatments were different for normal or non-normal distributions respectively. Male and female infections were analyzed separately so that the effect of treatment on fruit production could be assessed. Variables that were significantly different as a result of treatment effect were plotted and trends were observed by calculating means. All statistical calculations were conducted using the software package SigmaStat 2.03 (SPSS Inc., Chicago, IL).

### 7.2.3 Total shoot removal

In a separate experiment, the effect of shoot removal from *A. americanum* by a biological control agent was estimated by manually removing all of the shoots from *A. americanum* infections and comparing them with non-damaged controls. This experiment was conducted to estimate the time to new fruit production following

biological control treatment that removed all shoots. The *A. americanum* infections selected were all localized infections located on small lodgepole pine trees. Each treated infection was paired with a control, from which no shoots were removed. Although the primary variable of interest was the number of female fruit, other variables that related to the growth of dwarf mistletoe were also recorded; therefore, male and female infections were selected. The experiment was replicated near Canal Flats (50° 7.659' N 116° 0.405' W 1081 m) and Lytton (50° 29.310' N 121° 36.200' W 1291 m), British Columbia. Prior to treatment, the number of shoots, maximum shoot height, number of fruit (female), *A. americanum* swelling diameter and length were recorded. Twenty female infections were selected at both sites, while twenty and ten male infections were selected at Canal Flats and Lytton, respectively. Infections of the same sex were paired based on the number of shoots and one of the infections was randomly selected, by tossing a coin, to have all shoots removed. The experiment was established at both locations in August 1998 and was assessed in August of 1999, 2000 and 2001, prior to seed dispersal. Data collected at the assessments was the same as the pretreatment data and included the number of buds in 2000 and 2001, and the number of pollinated female flowers in 2001. Male and female infections, as well as sites, were analyzed independently. Analysis of variance and analysis of variance on ranks (if the assumptions of a normal distribution or equal variance were not met) was used to detect any differences between treatments using the statistical package SigmaStat (SPSS Inc., Chicago, IL).

### 7.3 Results

#### 7.3.1 Partial shoot removal experiment

At the 1-year assessment, no replicates were dead, but at the 2-year assessment of the experiment, 13 replicates were found to be dead, 11 of which were female (Table 7-2). Death of the dwarf mistletoe was not a result of treatment application; rather, it was the result of death of the host branch.

Table 7-2. Mortality two years after treatment.

Treatment (% removed)	Female	Male
1 (25%)	2	1
2 (50%)	3	1
3 (75%)	1	0
4 (cut)	2	0
5 (0%)	2	0
6 (100%)	1	0

##### 7.3.1.1 Effects of treatment on growth of the endophytic system

Prior to treatment application, there were no significant differences between the swelling diameter or length of *A. americanum* infections assigned to each treatment. Throughout the course of the experiment, *A. americanum* swelling diameter and length were not significantly different between treatments; treatment application had no effect on the growth of the endophytic system. Swelling diameter and length increased throughout the course of the experiment (Figure 7-1).

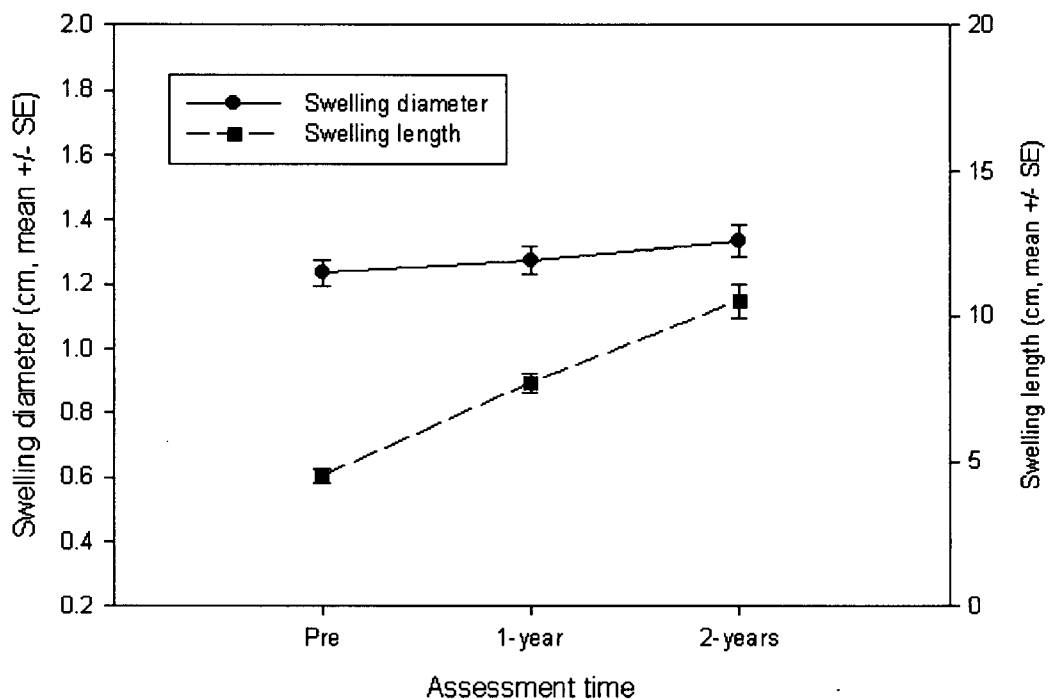


Figure 7-1. Change in *A. americanum* swelling diameter and length over the course of the partial shoot removal experiment. Treatments pooled. Mean +/- standard error of the mean.

### 7.3.1.2 New shoot production

Following treatment application, the production of buds was quantified as a measure of the production of new shoots. There was no significant difference between treatments with respect to bud production on male or female *A. americanum* swellings one or two years after treatment due to high variability (Figure 7-2). Treatment 6, 100% shoot removal, resulted in the production of the most buds two years following treatment, but it was not significantly different from the other treatments. The buds present at each assessment are representative of bud production during that year, there was no carry over of buds from 1-year to 2-years because the growth rate of the buds was fast enough that buds were classified as shoots in the following year.

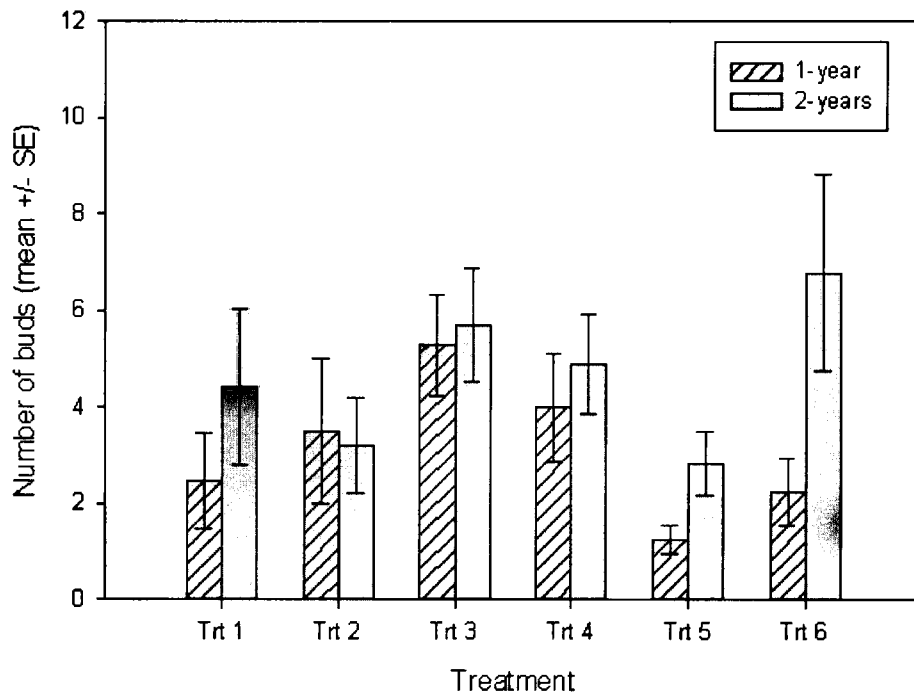


Figure 7-2. Bud production one and two years after treatment. Male and female infections pooled. Mean number of buds per *A. americanum* infection +/- standard error of the mean.

The number of female shoots was not significantly different prior to, or following, treatment over the course of the experiment. The maximum shoot length of female *A. americanum* subjected to treatment 6 was significantly less ( $p=0.009$ ) than treatment 1, but not the other treatments and there was no difference at 2-years.

The number of male shoots were also not significantly different prior to treatment application or two years later, but one year after treatment, *A. americanum* subjected to treatment 2 had significantly more ( $p=0.002$ ) shoots than *A. americanum* subjected to treatment 6. The maximum shoot length of male infections was not significantly different prior to treatment, or after two years, but at 1-year, treatments 2 and 3 had significantly longer ( $p<0.001$ ) shoots than infections treated with treatment 6.

### 7.3.1.3 Fruit production

Average fruit production on *A. americanum* swellings of each treatment at 0-, 1- and 2-years following treatment application is shown in table 7-3. Fruit production one year following treatment application was significantly different ( $p=0.009$ ) between treatments 1 and 6, 25% and 100% shoot removal respectively, but not significantly different between treatments at 2-years. Fruit produced in the year after treatment were a result of pollinated flowers that were present at the time of treatment application, while fruit produced in the second year were a result of flowers produced in the year after treatment. Generally, the total number of fruit present declined throughout the experiment (Table 7-3), and the average number of fruit per shoot declined as well (Figure 7-3).

Table 7-3. Partial shoot removal experiment. Average number of fruit present on female *A. americanum* infections of each treatment at every assessment and average number of flowers present at the final assessment.

Treatment (% removed)	Pre treatment	1-year	2-years	Flowers, 2-years
1 (25%)	60	34	7	40
2 (50%)	105	7	6	64
3 (75%)	54	26	3	58
4 (cut)	66	9	10	118
5 (0%)	100	45	21	18
6 (100%)	204	0	0	13

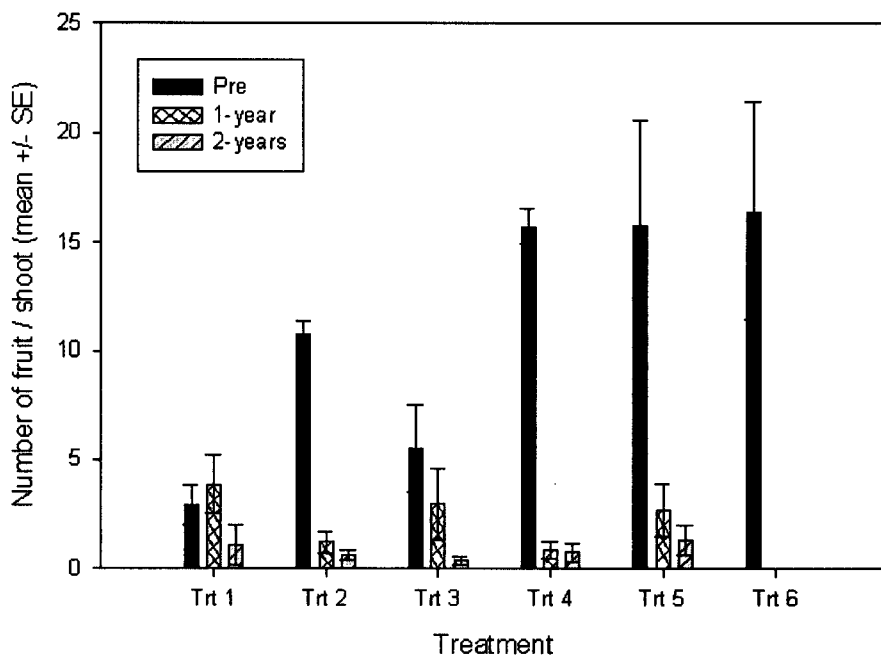


Figure 7-3. Average number of fruit per shoot over the course of the experiment for every treatment. Mean +/- standard error of the mean.

When the number of fertilized female flowers was counted as a predictor of fruit production three years after treatment, there was no significant difference between *A. americanum* infections treated with the different treatments. Flower production two years following treatment application suggests that the fruit crop three years after treatment will be larger than the previous years for all treatments except treatment 5, which appears to be declining (Table 7-3). As with fruit production, flower production was highly variable amongst replicates of the same treatment; some *A. americanum* replicates had many flowers while others had zero.

#### 7.3.1.4 Cut shoots

The cut shoot treatment was conducted to determine how *A. americanum* responded to dieback of the top portion of the shoot. When the shoots were cut, the cut segment died back to the proximal node and abscised at the node, while the remainder of the shoot appeared unaffected. Fruit production on the unaffected portions of the shoots continued and fruit were produced one year following treatment application.

### 7.3.1.5 Control

The control for this experiment was treatment 5, zero shoot removal. Over the course of the experiment, the number of shoots, maximum shoot length and number of fruit declined, with the greatest decrease over the period of the first year, suggesting there was a general decline in the stand (Figure 7-4). The number of fruit was highly variable amongst replicates of the female control. The swelling diameter and length increased over the course of the experiment as it did with *A. americanum* infections of all treatments.

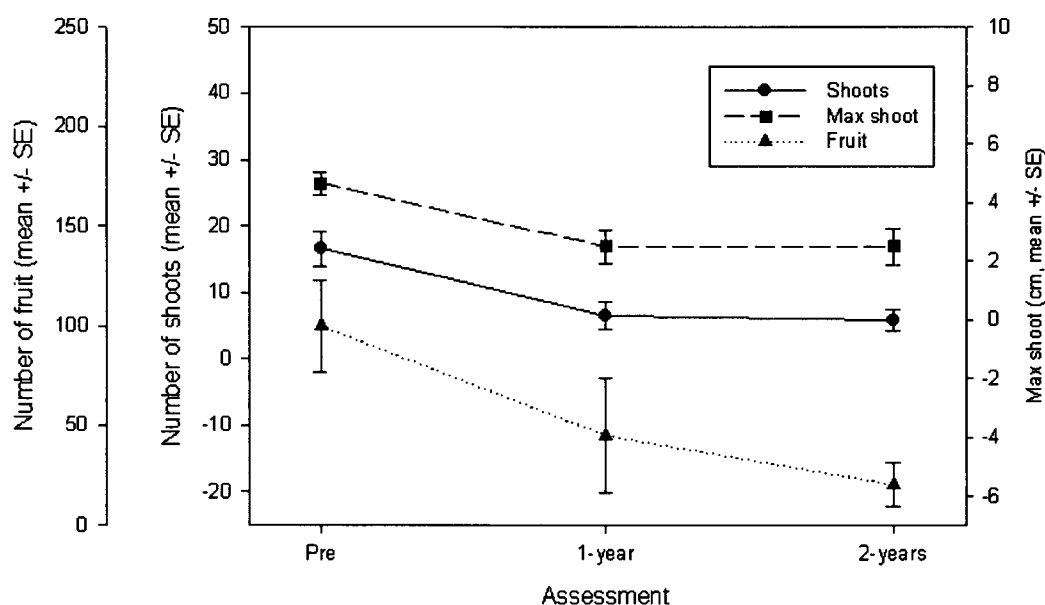


Figure 7-4. Observation of control *A. americanum* infections. Number of shoots and maximum shoot length of male and female infections pooled. Mean +/- standard error of the mean for all plots.

### 7.3.2 Total shoot removal

The effect of a biological control agent that removed all of the aerial shoots of *A. americanum*, but did not affect the endophytic system, was mimicked by manually removing all shoots and buds from *A. americanum* swellings and comparing the treated swellings with untreated controls. Results are summarized in table 7-4.

*Arceuthobium americanum* mortality occurred during the course of the experiment, through a combination of squirrel damage, normal self-pruning and disease

by *Colletotrichum gloeosporioides*. During the assessment period, *C. gloeosporioides* was isolated from the shoots of 3 female and 4 male dwarf mistletoe infections at Canal Flats; all but 1 female were replicates of the control. Mortality was lower at Lytton, resulting in the death of 4 female infections; 3 of which were located on branches that died after treatment and 1 that was on a branch that was broken off. Although mortality occurred during the experiment, it was spread out over the treatments and when the results were pooled, Chi square indicated that mortality was independent of treatment.

Table 7-4. Average response of each variable measured up to 3 years following treatment application at both experimental locations. All measurements are recorded in centimeters. Trt = treatment, Ck = control.

Location	Sex	Variable	Pre		1-year		2-years		3-years	
			Trt	Ck	Trt	Ck	Trt	Ck	Trt	Ck
Canal	Female									
Flats		Diameter	1.37	1.7	1.27	1.81	1.38	1.55	1.41	1.61
		Length	3.70	4.45	4.67	4.94	8.11	7.37	9.83	9.15
		# shoots <sup>†</sup>	20	18	4	7	6	10	7	13
		Max shoot	4.6	4.85	0.6	2.32 <sup>§</sup>	1.4	2.07	2.20	1.97
		# buds	-- <sup>‡</sup>	--	--	--	8	7	2	5
		# fruit	54	16 <sup>§</sup>	0	20	0	0	4	6
		# flowers	--	--	--	--	--	--	50	69
		Mortality	0	0	0	1	3	2	3	2
	Male									
		Diameter	1.39	1.47	1.14	1.59	1.19	2.11	1.26	1.97
		Length	3.90	3.55	3.94	4.57	6.62	7.01	8.76	9.22
		# shoots <sup>†</sup>	15	17	12	26	5	9	2	12 <sup>§</sup>
		Max shoot	6.15	6.16	1.04	6.17 <sup>§</sup>	1.23	3.27 <sup>§</sup>	1.58	2.48
		# buds	--	--	--	--	5	9	6	12
		Mortality	0	0	1	3	1	3	2	6

Location	Sex	Variable	Pre		1-year		2-years		3-years	
			Trt	Ck	Trt	Ck	Trt	Ck	Trt	Ck
Lytton	Female									
		Diameter	1.07	1.29	0.96	1.28 <sup>§</sup>	1.12	1.47	1.06	1.55 <sup>§</sup>
		Length	2.58	3.55	3.33	3.99	6.37	9.26 <sup>§</sup>	6.52	10.54 <sup>§</sup>
		# shoots <sup>†</sup>	14	12	0	18 <sup>§</sup>	8	6	10	14
		Max shoot	3.86	3.94	0	3.83 <sup>§</sup>	1.60	2.51 <sup>§</sup>	1.86	2.02
		# buds	--	--	--	--	4	13	9	18
		# fruit	60	50	0	65 <sup>§</sup>	2	5	2	5
		# flowers	--	--	--	--	--	--	39	82
		Mortality	0	0	0	0	3	1	3	1
	Male									
		Diameter	0.98	1.00	1.02	1.06	0.93	1.63	1.02	1.04
		Length	3.60	3.10	4.40	3.50	7.14	9.49	8.37	9.10
		# shoots <sup>†</sup>	13	14	0	10	12	4	18	9
		Max shoot	4.06	4.72	0	5.70 <sup>§</sup>	0.92	3.05	2.54	2.93
		# buds	--	--	--	--	5	7	13	13
		Mortality	0	0	0	0	0	0	0	0

<sup>†</sup> One year following treatment application, buds were not distinguished from shoots, therefore, the total number of shoots at 1-year includes buds.

<sup>§</sup> treatment is significantly different from the control by ANOVA with  $\alpha = 0.05$ .

<sup>‡</sup> Data not recorded.

### 7.3.2.1 Fruit production

The time to reestablishment of fruit production, and fruit production on the controls, was quantified by counting the number of fruit at each assessment. Prior to treatment, the *A. americanum* infections designated to be treated had significantly ( $p=0.043$ ) more fruit than the controls at Canal Flats, even though treatment and control were randomly assigned. This occurred because infections were paired based on the number of shoots, not the number of fruit. Following treatment application, there was no

significant difference in fruit production between the treatment and control *A. americanum* infections at every assessment at Canal Flats. At Lytton, there was no significant difference between *A. americanum* infections designated as treatment or controls prior to treatment application, but one year after treatment, the number of fruit at Lytton was significantly ( $p=0.004$ ) higher on the controls than the treated infections. Two and three years after treatment, there was no significant difference in the number of fruit between the treatment and control.

There was no significant difference between the treatment and control two or three years after treatment at Canal Flats or Lytton because fruit production on the control *A. americanum* infections decreased dramatically, not because fruit production on the treated infections increased. Prior to treatment all female infections at Canal Flats bore fruit, while at 1 year, 4 of 10 control infections bore fruit, but the difference between the treatment and control was not significant, and at 2 years only 1 fruit was present on 1 of 8 control infections. Three years after treatment, 2 of 8 control infections and 2 of 7 treated infections bore fruit. At Lytton, fruit production was greatly reduced two years after treatment. All control infections bore fruit in the year following application, while 4 of 9 control infections at 2 years and 2 of 9 control infections at 3 years bore fruit. Of the treated infections at Lytton, none produced fruit the year following shoot removal, while 1 of 7 infections produced consecutive fruit crops at 2 and 3 years after treatment.

The production of consecutive crops of *A. americanum* fruit on control infections at Canal Flats and Lytton are recorded in table 7-5. One control infection at Canal Flats bore fruit prior to treatment and then again three years after treatment. After 2 consecutive crops of fruit, two infections died at Canal Flats and one infection died at Lytton.

Table 7-5. Number of control replicates of *A. americanum* in each category to record consecutive crops of fruit at Canal Flats and Lytton.

Site	1 crop	2 crops	3 crops	4 crops	Discontinuous
Canal Flats	5	3	0	1	1
Lytton	1	5	3	1	0

The number of pollinated female flowers was counted three years after treatment as a measure of potential fruit production four years following treatment. At Canal Flats, 3 of 8 controls and 4 of 7 treated infections had pollinated female flowers and at Lytton, 5 of 9 controls and 5 of 7 treated infections had pollinated female flowers. The number of flowers was not significantly different between treatments, and the average number of flowers on each infection suggests that fruit production will be high four years after treatment.

#### 7.3.2.2 Endophytic system growth

Growth of the endophytic system was not significantly different between treatments at either site or for either sex. Treatment application did not affect endophytic system growth, the average diameter and length of the dwarf mistletoe swelling increased throughout the course of the experiment at both sites. The average increase in swelling diameter and length over the assessment period was 1 mm and 5.6 cm, respectively, at both sites.

#### 7.3.2.3 Shoot production

The number of shoots per swelling was not significantly different prior to treatment application. After treatment application, there was no significant difference in the number of male or female shoots at any time post treatment at Canal Flats or male shoots at Lytton. The number of female shoots was significantly greater on the controls compared to the treated infections one year after treatment at the Lytton site ( $p=0.004$ ), in years 2 and 3, there were no significant differences. A decrease in the number of shoots on the control, coupled with an increase in the number of shoots on treated *A. americanum* swellings, accounted for the finding of no significant difference.

One year after treatment, buds were not distinguished from shoots; therefore, the total number of shoots present at the 1-year assessment included buds and buds cannot be separated from the number of shoots. The production of new shoots was quantified two and three years following treatment by counting the number of buds and it was found that there was no significant difference between treatments, sexes or sites.

The female maximum shoot length of control infections at Canal Flats was significantly longer than the treated infections at 1-year ( $p=0.043$ ). The male maximum shoot length of controls was significantly longer than treated infections at 1-year ( $p<0.001$ ) and 2-years ( $p=0.023$ ). Three years after treatment, there were no significant differences in maximum shoot length, as was the case prior to treatment. The maximum shoot length of female control infections at Lytton was significantly longer than the treated infections at 1-year ( $p<0.001$ ) and 2-years ( $p<0.001$ ), while the male controls were significantly longer than the treated infections at 1-year ( $p<0.001$ ).

## **7.4 Discussion**

The partial shoot removal experiment was designed to mimic the effect of a biological control treatment that did not result in one hundred percent shoot death. The total shoot removal experiment was conducted to estimate the time to new fruit production following biological control treatment that killed all of the aerial shoots but not the endophytic system. Mortality and high variability between replicates of the same treatment affected the results of the experiments; however, general conclusions can be drawn from the response of the dwarf mistletoe to treatment.

### **7.4.1 Endophytic system growth**

No decrease in the growth rate of the endophytic system, as estimated by change in the length and diameter of the *A. americanum* swelling, was noted following treatment application. It was hypothesized that endophytic system growth rate would slow because the nutrient sink generated by *A. americanum* (Clark and Bonga, 1970) would be reduced following removal of the aerial shoots; however, the null hypothesis of no significant difference was not rejected. To maintain connectivity between the sinkers in the xylem and the cortical strands in the phloem, meristematic activity must occur within the sinker

tissue as new xylem and phloem are laid down by the vascular cambium (Hunt et al., 1996). The finding of no change suggests that the growth of the endophytic system may be regulated or influenced by the growth of the host branch.

#### **7.4.2 New shoot production**

The production of buds was not found to be significantly different between treatments following treatment application in the partial shoot removal or the total shoot removal experiments. The production of buds occurs via meristematic activity on the surface of the outer cortical strand, which ultimately results in emergence of the bud from the bark (Cohen, 1954). New shoots are produced at the margin of the endophytic system as the endophytic system expands, suggesting that the production of buds may be regulated by expansion of the endophytic system. Although the production of new buds was not significantly different between treatments in these studies, bud production was found to be significantly increased following treatment 2 application in the field trial that was described in Chapter 3. The variability of individual replicates observed in these studies was high and with increased sample size, significant differences may have been noted, but the trend observed suggests that *A. americanum* does increase new shoot production following treatment.

#### **7.4.3 Fruit production**

Fruit production one year after treatment application was a result of immature fruit present on the shoots at the time of treatment, while fruit production in the following years was a result of flowers that were produced on the remaining shoots. There was a sharp decrease in fruit production in both treatments and controls in both experiments two years after treatment. The number of flowers present in the second year suggested that the seed crop will be higher in the following season, assuming each pollinated flower will produce a viable seed. The number of pollinated flowers present in the year following treatment was not counted; therefore, it is impossible to determine if all flowers counted in the second year will develop to maturity. It is unlikely that every pollinated flower counted will survive to produce a viable seed; of the 93% of *A. tsugense* shoots that bore flowers, only 43% produced fruit prior to dying (Smith, 1977). Variability

amongst replicates of the same treatments was high with respect to fruit and flower production. The general decline in the number of fruit present, as well as the variability between replicates of the same treatment, prevent a definitive conclusion with respect to changes in fruit production following treatment application.

#### **7.4.4 Response of shoots to dieback from the tip**

Trimming the shoot at the midpoint mimicked the effect of a biological control agent that resulted in death of the top portion of the shoot. When the shoot was cut, the segment died back to the proximal node and fell off. Shoot development carried on normally and flowers that were present on the laterals developed into fruit. An increase in the production of lateral branches on the shoots below the cut was not quantified; therefore, it is impossible to determine if *A. americanum* responded by increasing lateral branches. The production of new buds was no different from the other treatments, and fruit production declined in the following years as it did with all other treatments.

#### **7.4.5 Shoot characteristics over time**

The controls of both experiments were utilized to test the hypothesis that *A. americanum* infection characteristics do not change over time. Although the number of shoots remained constant, the maximum shoot height and number of fruit decreased. This suggests that *A. americanum* infections declined over time. Observation of *A. americanum* in the field suggests that the first shoots that are produced by the infection are vigorous and produce large crops of fruit. Individual shoots cannot be aged; however, under controlled conditions, individual shoots could be followed and their life history could be determined, providing an understanding of the number of consecutive crops of fruit they could bear.

The health of the *A. americanum* infection is directly related to the health of the individual branch upon which it is located. As lodgepole pine increases in height, shade increases in the lower canopy. As the lower canopy becomes shaded, the branches die, effectively raising the living crown as the tree grows in height (Koch, 1996). Mortality of the host branch over the term of the experiment occurred in all experiments that were conducted in this study. The decline of the host branch associated with increased shading

and natural pruning of lodgepole pine has likely resulted in the decrease in *A. americanum* vigour over the course of the experiment.

#### 7.4.6 Biological control treatment periodicity

The total shoot removal experiment was established so that the time to new shoot production following biological control that removed all shoots, without damaging the endophytic system could be estimated. At Lytton, fruit were observed on shoots that had 100% shoot removal two years earlier. Seed development requires 16 months in *A. americanum*. For fruit to be produced two years after treatment, a shoot would need to grow and set flowers in the year following treatment application. The treatment was applied in August of 1998; it is highly doubtful that shoots grew and produced flowers that were pollinated in the spring of 1999. It is probable that the observation of fruit two years following treatment was the result of a shoot or bud that was accidentally missed during treatment application. This is supported by the fact that no fruit were observed on the 100% shoot removal treatment of the partial shoot removal experiment or at Canal Flats. Flowers were recorded two years after treatment on replicates of the 100% shoot removal treatment in the partial shoot removal experiment, suggesting that seed will be shed in the fall of the third year after treatment. Fruit were observed three years after shoot removal at Canal Flats and Lytton. When *A. americanum* was treated with ethephon, an ethylene releasing compound that causes shoot abscission, shoots bearing fruit were noted for 3% of infections two years after treatment, increasing to 52% of infections after 5-years (Nicholls, 1988). These data suggest that the recovery of *A. americanum* following shoot abscission is variable, perhaps as a result of host branch vigour and light availability, but that biological control reapplication may be required as soon as three years following the initial treatment.

Consecutive crops of fruit on individual shoots of *A. americanum* have been observed for up to five years (Hawksworth and Wiens, 1996) and multiple crops of seeds over successive years have been observed on *A. tsugense* and *A. laricis* (Smith 1977). In this study, individual shoots were not followed, but consecutive crops of fruit were observed on some infections for at least four years at Canal Flats and Lytton. If a biological control agent were able to remove the aerial shoots of *A. americanum*, seed

dispersal onto the regenerating stand would be significantly reduced for a period of at least 3 years and the age class distribution of shoots would be interrupted.

#### 7.4.7 Conclusions

Mortality, high variability and declining host branch vigour affected the results of this study. The impact of partial shoot removal on fruit production could not be assessed satisfactorily. Growth of the endophytic system did not change as a result of treatment, indicating that this characteristic may be closely related to growth of the host branch. Bud production did increase following total shoot removal in these experiments, and in the field trial in Chapter 3; however, the difference was not significant.

Treatment application was designed to mimic the mode of action of a biological control agent; however, physically cutting the aerial shoots may induce a different host response than disease caused by *C. gloeosporioides*. Without inoculating *A. americanum* with *C. gloeosporioides*, it is difficult to determine exactly how the dwarf mistletoe responds to infection, but the wounds applied to *A. americanum* may give a minimum response time. Alternative methods of determining the maximum treatment interval include treatment with Ethephon and closely observing the development of individual shoots over time.

From a biological control perspective, the results indicate that all shoots must be removed to cause a significant reduction in seed production, and that the maximum period of time between biological control applications necessary to achieve effective dwarf mistletoe control would be 3 years.

## Chapter 8 – Conclusions

Research in this study was focused toward the development of an inundative biological control strategy for lodgepole pine dwarf mistletoe. Previous studies into biological control of dwarf mistletoes were directed at establishing a self-regulating population of the biological control agent under the classical biological control paradigm. The development of an inundative biological control approach may provide an alternative dwarf mistletoe management tool to minimize establishment of dwarf mistletoe in regenerating stands.

The initial stage of the study was directed at collecting diseased *A. americanum* for the isolation of fungi that may be potential inundative biological control agents. *Colletotrichum gloeosporioides* was collected throughout the region surveyed and *Caliciopsis arceuthobii* was observed but not isolated. Other fungi, including *Cladosporium sp.* and *Schlerophoma pithyophita* were also isolated. Lodgepole pine were inoculated with *A. americanum* under greenhouse conditions to serve as experimental units for testing under controlled conditions, however, *A. americanum* infections had not emerged in time for any portions of the research conducted in this study. The lack of experimental units to test fungi isolated from diseased *A. americanum* that were collected in the survey resulted in the selection of *C. gloeosporioides* as the candidate organism because it met at least three of the six criteria of a dwarf mistletoe biological control agent as set out by Wicker and Shaw (1968). The distribution of the fungus coincided with the range of *A. americanum* that was surveyed, it produced abundant inoculum in culture and the field, and it has an efficient mode of curtailing *A. americanum* development. Further study of the infection process and an assessment of virulence are likely to confirm that *C. gloeosporioides* meets these criteria as well. Additionally, *C. gloeosporioides* was readily cultured and inoculum was easily generated in culture. Although *C. arceuthobii* has an efficient mode of reducing *A. americanum* spread and intensification, it was not selected because it was not cultured and inoculum has not been generated in culture in other studies. Without experimental units under controlled conditions, isolate selection was made based on the growth and sporulation characteristics of four different isolates. The isolate that produced the most conidia in culture and had the best growth over a wide range of temperatures was selected. Initially

a sodium alginate – kaolin clay formulation was tested in the greenhouse on experimental units of *A. tsugense* and this formulation was found to be unsatisfactory.

The selected isolate was then formulated in 'Stabileze' and inoculated onto *A. americanum* under field conditions near Lytton, British Columbia. The results of successful establishment on a small number of replicates indicated that when *C. gloeosporioides* becomes established, it is able to cause extensive damage to *A. americanum*. A successful inundative biological control agent for *A. americanum* must interfere with seed production to prevent spread to uninfected trees and intensification in infected trees. There was no significant reduction in seed production as a result of inoculation with *C. gloeosporioides* in the field trial. This may be due to low inoculum concentration in the formulation, low virulence of the selected isolate, or the environmental conditions at the field site may have been limiting.

It has been reported that *C. gloeosporioides* has been observed in the endophytic system of *A. americanum*. From a biological control perspective, this would likely result in increased time between treatment application because new shoot production would be inhibited. To study the relationship between *C. gloeosporioides* and *A. americanum*, naturally diseased and inoculated *A. americanum* swellings were cultured and examined microscopically. In this study, it was found that *C. gloeosporioides* was restricted to the basal cup region of *A. americanum* and did not infect the endophytic system. This may be indicative of a difference between this pathosystem and the other pathosystems that have been investigated.

The natural distribution of *C. gloeosporioides* in a stand of *A. americanum* infected lodgepole pine was assessed to predict establishment success of *C. gloeosporioides* at different crown positions following inoculation. It was found that *A. americanum* at all crown positions was equally susceptible to *C. gloeosporioides* indicating that if the inoculum contacts the dwarf mistletoe, probability of establishment is the same at all canopy positions.

The study designed to follow *Caliciopsis arceuthobii* infection of *A. americanum* indicated that the fungus caused a significant reduction in fruit production and that it was able to infect *A. americanum* that was disease free in previous years. The difficulties of

inoculum production and field inoculation require extensive research before this fungus can be used as an inundative biological control agent for *A. americanum*.

To study the response of *A. americanum* to biological control treatment and to determine the treatment interval required to prevent *A. americanum* infection of a regenerating stand, two separate experiments were conducted. The rate of endophytic system growth was not related to the amount of damage that was inflicted upon the aerial system, suggesting that endophytic system growth may be regulated by the host. There was not a significant increase in bud production following total shoot removal, however, the trend observed indicates that bud production may be increased following shoot removal. The time to fruit production following total shoot removal was three years, suggesting that the maximum treatment interval necessary to prevent seed dispersal, assuming no damage to the endophytic system, is three years. Future studies should be conducted on *A. americanum* so that fruit production over the lifespan of the dwarf mistletoe is better understood. It was obvious that *A. americanum* fruit production on individual infections varied from year to year. If the source of this variation can be determined, the biological control treatment periodicity may be extended.

The results of these studies suggest that *C. gloeosporioides* has potential as an inundative biological control agent for *A. americanum* due to the damage that it causes when it becomes successfully established and that *A. americanum* at all crown positions was found to be susceptible to infection. Several challenges must be overcome before a successful inundative biological control strategy is developed. The optimum conditions for fungal establishment were not assessed because no experimental units were ready for these studies. Successful establishment of *A. americanum* on the lodgepole pine that were inoculated at the start of this study is now becoming evident, therefore, experimental units that can be placed under controlled conditions to study the infection process of *C. gloeosporioides* are now available. These experimental units can also be used for the selection of the most virulent isolate of *C. gloeosporioides*, as well as assessing the pathogenicity of other fungi that were collected during the collection phase. If the necessary conditions required for *C. gloeosporioides* establishment can be determined in the greenhouse, field inoculation can be timed to coincide with as close to optimum conditions as are available. If inoculation conditions can be optimized, frequent

reapplication is required, as demonstrated by the shoot removal experiment. The role of secondary inoculum production should also be studied, as secondary inoculum may serve to intensify *C. gloeosporioides* infection as well as increase the period of time necessary for preventing seed production. Another challenge that must be overcome is inoculation of *A. americanum* in the upper crown. Upper crown infections are the most important from a spread and intensification standpoint, and for biological control to be successful they must be inoculated. When applied to the upper crown by helicopter, Ethepon was ineffective in controlling dwarf mistletoe (Baker et al., 1989; Robbins et al., 1989), suggesting that considerable study will be required to develop an application system that effectively inoculates *A. americanum* in the upper crown.

The primary objective of this research was to investigate the possibility of developing an inundative biological control strategy for *A. americanum*. Focusing the study on *C. gloeosporioides* provided important insight into what is required for a successful biological control strategy for *A. americanum*. The data collected in this study suggests that considerable research relating to conditions necessary for establishment of the fungus, improved efficacy and field inoculation techniques are required to develop *C. gloeosporioides* into a successful biological control agent. If these challenges to development cannot be overcome, then the use of *C. gloeosporioides* as an inundative biological control agent for *A. americanum* is unlikely, but further study should be conducted before this approach is abandoned. If the results of future research suggest that the *C. gloeosporioides* provides adequate control of *A. americanum*, future study of the genetic stability of *C. gloeosporioides*, as well as host range studies will need to be completed before the fungus can be used commercially as an inundative biological control agent for *A. americanum*.

## Literature Cited

- Alosi, M.C., and Calvin, C.L. 1984. The ultrastructure of dwarf mistletoe (*Arceuthobium* spp.) sinker cells in the region of the host secondary vasculature. *Canadian Journal of Botany* 63: 889-898.
- Alosi, M.C., and Calvin, C.L. 1984b. The anatomy and morphology of the endophytic system of *Arceuthobium* spp. In *Biology of dwarf mistletoes: proceedings of the symposium*. Edited by F.W. Hawksworth and R.F. Scharpf. U.S. Dep. Agric. For. Serv. Rocky Mount. Stn., Gen. Tech. Rep. RM-111. pp. 40-52.
- Anonymous. 1995. Dwarf mistletoe management guidebook. Forest Practices Code of British Columbia. Province of British Columbia. Ministry of Forests. 20 p.
- Auld, B.A., and Morin, L. 1995. Constraints in the development of bioherbicides. *Weed Technology* 9: 638-652.
- Baranyay, J.A. 1968. Squirrel feeding on dwarf mistletoe infections. *Bi-Monthly Progress Report*. Ottawa, Canada Department of Forestry 24(5): 41-42.
- Baranyay, J.A., and Smith, R.B. 1972. Dwarf mistletoes in British Columbia and recommendations for their control. Canadian Forest Service, Pacific Forest Research Centre. Report BC-X-72. 18 p.
- Bailey, J.A., O'Connell, R.J., Pring, R.J., and Nash, C. 1992. Infection strategies of *Colletotrichum* species. Pages 88-120 in: *Colletotrichum: Biology, Pathology and Control*. J.A. Bailey and M.J. Jeger, eds. CAB International, Wallingford, UK.
- Bailey, J.A., Nash, C., Morgan, L.W., O'Connell, R.J., and TeBeest, D.O. 1996. Molecular taxonomy of *Colletotrichum* species causing anthracnose on the Malvaceae. *Phytopathology* 86: 1076-1083.
- Barnett, H.L., and Hunter, B.B. 1998. *Illustrated genera of imperfect fungi*, 4<sup>th</sup> edition. APS Press, St. Paul, MN. 218 p.
- Bhandari, N.N., and Nanda, K. 1970. The endophytic system of *Arceuthobium minutissimum*, the Indian dwarf mistletoe. *Annals of Botany* 34: 517-525.
- Biring, B.S., Comeau, P.G., and Boateng, J.O. 1996. Effectiveness of forest vegetation control methods in British Columbia. Canadian Forest Service and British Columbia Forest Service, FRDA Handbook 011. 51 p.
- Boyette, C.D. 1994. Unrefined corn oil improves the mycoherbicidal activity of *Colletotrichum truncatum* for hemp sesbania (*Sesbania exaltata*) control. *Weed Technology* 8: 526-529.

- Boyette, C.D., Quimby, P.C., Jr., Bryson, C.T., Egley, G.H., and Fulgham, F.E. 1993. Biological control of hemp sesbania (*Sesbania exaltata*) under field conditions with *Colletotrichum truncatum* formulated in an invert emulsion. *Weed Science* 41: 497-500.
- Boyette, C.D., Quimby, P.C.Jr., Caesar, A.J., Birdsall, J.L., Connick, W.J.Jr., Daigle, D.J., Jackson, M.A., Egley, G.H., and Abbas, H.K. 1996. Adjuvants, formulations, and spraying systems for improvement of mycoherbicides. *Weed Technology* 10: 637-644.
- Broshot, N.E., and Tinnin, R.O. 1986. The effect of dwarf mistletoe on starch concentration in the twigs and needles of lodgepole pine. *Canadian Journal of Forest Research* 16: 658-660.
- Carrington, M.E., Roberts, P.D., Urs, N.V.R.R., McGovern, R.J., Seijo, T.E., and Mullahey, J.J. 2001. Premature fruit drop in saw palmettos caused by *Colletotrichum gloeosporioides*. *Plant Disease* 85: 122-125.
- Cerkauskas, R.F. 1988. Latent colonization by *Colletotrichum* spp.: Epidemiological considerations and implications for mycoherbicides. *Canadian Journal of Plant Pathology* 10: 297-310.
- Chapman, B., and Xiao, G. 2000. Inoculation of stumps with *Hypholoma fasciculare* as a possible means to control armillaria root disease. *Canadian Journal of Botany* 78: 129-134.
- Clark, J., and Bonga, J.M. 1970. Photosynthesis and respiration in black spruce (*Picea mariana*) parasitized by eastern dwarf mistletoe (*Arceuthobium pusillum*). *Canadian Journal of Botany* 48: 2029-2031.
- Cohen, L.I. 1954. The anatomy of the endophytic system of the dwarf mistletoe, *Arceuthobium campylopodum*. *American Journal of Botany* 41: 840-847.
- Dobie, J., and Britneff, A.A. 1975. Lumber grades and volumes from lodgepole pine infected with dwarf mistletoe. *Wood and Fiber* 7: 104-109.
- Dowding, S.E. 1931. *Wallrothiella arceuthobii*, a parasite of the Jack-pine mistletoe. *Canadian Journal of Research* 5: 219-230.
- Dumas, B., Centis, S., Sarrazin, N., and Esquerré-Tugayé, M. 1999. Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Applied and Environmental Microbiology* 65: 1769-1771.

- Egley, G.H. and Boyette, C.D. 1995. Water-corn oil emulsion enhances conidia germination and mycoherbicidal activity of *Colletotrichum truncatum*. *Weed Science* 43: 312-317.
- Ellis, D.E. 1939. A fungus disease of *Arceuthobium*. *Phytopathology* 29: 995-996.
- Ellis, D.E. 1946. Anthracnose of dwarf mistletoe caused by a new species of *Septogloeum*. *Journal of the Elisha Mitchell Science Society* 62: 25-50.
- Estrada, A.B., Dodd, J.C., and Jeffries, P. 2000. Effect of humidity and temperature on conidial germination and appressorium development of two Philippine isolates of the mango anthracnose pathogen *Colletotrichum gloeosporioides*. *Plant Pathology* 49: 608-618.
- Geils, B.W., and Mathiasen, R.L. 1990. Intensification of dwarf mistletoe on southwestern Douglas-fir. *Forest Science* 36: 955-969.
- Gilbert, J.A. 1984. The biology of dwarf mistletoes (*Arceuthobium* spp.) in Manitoba. M.Sc. Thesis, Department of Botany, University of Manitoba. 138p.
- Gill, L.S. 1935. *Arceuthobium* in the United States. *Connecticut Academy of Arts and Science Transactions* 32: 111-245.
- Gill, L.S. 1952. A new host for *Septogloeum gillii*. *Plant Disease Reporter* 36: 300.
- Hadfield, J.S., Mathiasen, R.L., and Hawksworth, F.G. 2000. Forest insect and disease leaflet 54, Douglas-fir dwarf mistletoe. United States Department of Agriculture Forest Service. 10p.
- Harper, G.J., Comeau, P.G., Hintz, W., Wall, R.E., Prasad, R., and Becker, E.M. 1999. *Chondrostereum purpureum* as a biological control agent in forest vegetation management. II. Efficacy on sitka alder and aspen in western Canada. *Canadian Journal of Forest Research* 29: 852-858.
- Hasan, S. and Ayers, P.G. 1990. Tansley review no. 23. The control of weeds through fungi: principles and prospects. *New Phytologist* 115: 201-222.
- Hawksworth, F.G. 1972. Biological control of the mistletoes. In: Nordin, V.J., comp. *Biological control of forest diseases*. Ottawa, Canada. The Canadian Forestry Service. p. 83-92.
- Hawksworth, F.G. 1977. The 6-class dwarf mistletoe rating system. Gen. Tech. Rep. RM-48. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. 7p.
- Hawksworth, F.G., Dixon, C.S., and Krebill, R.G. 1983. *Peridermium bethelii*: a rust

- associated with lodgepole pine dwarf mistletoe. *Plant Disease* 67: 729-733.
- Hawksworth, F. G. and Johnson, D.W. 1989. Biology and management of dwarf mistletoe in lodgepole pine in the Rocky Mountains. Gen. Tech. Rep. RM-169. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. 38p.
- Hawksworth, F.G., Wicker, E.F., and Scharpf, R.F. 1977. Fungal parasites of dwarf mistletoes. Gen. Tech. Rep. RM-36. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station. 14p.
- Hawksworth, F.G., and Wiens, D. 1972. Biology and classification of dwarf mistletoes (*Arceuthobium*). Agriculture Handbook 401. Washington, DC: U.S. Department of Agriculture, Forest Service. 234p.
- Hawksworth, F.G., and Weins, D. 1996. Dwarf mistletoes: biology, pathology, and systematics. Agricultural Handbook 709. Washington, DC: U.S. Department of Agriculture, Forest Service. 410p.
- Heath, M.C. 2000. Advances in imaging the cell biology of plant-microbe interactions. *Annual Review of Phytopathology* 38: 443-459.
- Hinds, T.E., Hawksworth, F.G., and McGinnies, W.J. 1963. Seed discharge in *Arceuthobium*: A photographic study. *Science* 140: 1236-1238.
- Hinds, T.E., and Hawksworth, F.G. 1965. Seed dispersal velocity in four dwarf mistletoes. *Science* 148: 517-519.
- Hintz, W.E., Becker, E.M., and Shamoun, S.F. 2001. Development of genetic markers for risk assessment of biological control agents. *Canadian Journal of Plant Pathology* 23: 13-18.
- Holdenrieder, O., and Greig, B.J.W. 1998. Biological methods of control. In *Heterobasidion annosum* Biology, Ecology, Impact and Control. Edited by S. Woodward, J. Steinlid, R., Karjalainen and A. Hüttermann. Wallingford, Oxon. CAB International. pp. 235-258.
- Hunt, R.S., Owens, J.N., and Smith, R.B. 1996. Penetration of western hemlock, *Tsuga heterophylla*, by the dwarf mistletoe *Arceuthobium tsugense*, and development of the parasite cortical system. *Canadian Journal of Plant Pathology* 18: 342-346.
- Johansen, D.A. 1940. Plant Microtechnique. Ed. 1, McGraw-Hill, New York.
- Knutson, D.M., and Hutchins, A.S. 1979. *Wallrothiella arceuthobii* infecting *Arceuthobium douglasii*: culture and field inoculation. *Mycologia* 71: 821-828.

- Koch, P. 1996. Lodgepole Pine in North America. Wood Products Society, Madison, WI. 1096p.
- Kope, H.H., Shamoun, S.F., and Oleskevich, C. 1997. First report of *Colletotrichum gloeosporioides* on *Arceuthobium tsugense* subsp. *tsugense* in Canada. Plant Disease 81: 1095.
- Kuijt, J. 1955. Dwarf mistletoes. The Botanical Review 21: 569-619.
- Kuijt, J. 1960. Morphological aspects of parasitism in the dwarf mistletoes (*Arceuthobium*). Publications in Botany 30. Berkeley, CA: University of California: 337-436.
- Kuijt, J. 1969. Observations on *Wallrothiella arceuthobii*, a fungus parasite of dwarf mistletoes. Canadian Journal of Botany 47: 1359-1365.
- Lennox, C.L., Morris, M.J., and Wood, A.R. 2000. Stumpout™ - Commercial production of a fungal inoculant to prevent re-growth of cut wattle stumps in South Africa. In Program and Abstracts of X International Symposium on Biocontrol of Weeds. July 4 – 14, 1999. Bozeman, Montana, U.S.A. Edited by N. Rush. Montana State University, Montana, U.S.A. p. 69.
- Leonard, O.A., and Hull, R.J. 1965. Translocation relationships in and between mistletoes and their hosts. Hilgardia 37: 115-153.
- Livingston, W.H., Brenner, M.L., and Blanchette, R.A. 1984. Altered concentrations of abscisic acid, indole-3-acetic acid, and zeatin riboside associated with eastern dwarf mistletoe infections on black spruce. In Biology of dwarf mistletoes: proceedings of the symposium. Edited by F.W. Hawksworth and R.F. Scharpf. U.S. Dep. Agric. For. Serv., Rocky Mount. Stn., Gen. Tech. Rep. RM-111. pp. 53-61.
- Lloyd, D., Angrove, K., Hope, G., and Thompson, C. 1990. A guide to site identification and interpretation for the Kamloops forest region, Part 1. British Columbia Ministry of Forests, Victoria, B.C., Land Management Handbook 23. p. 103-108.
- Lorang, J.M., Tuori, R.P., Martinez, J.P., Sawyer, T.L., Redman, R.S., Rollins, J.A., Wolpert, T.J., Johnson, K.B., Rodriguez, R.J., Dickman, M.B., and Ciuffetti, L.M. 2001. Green fluorescent protein is lighting up fungal biology. Applied and Environmental Microbiology 67: 1987-1994.
- Louis, I., and Cooke, R.C. 1985. Conidial matrix and spore germination in some plant pathogens. Transactions of the British Mycological Society 84: 661-667.

- Luo, Y., and TeBeest, D.O. 1999. Effect of temperature and dew period on infection of northern jointvetch by wild-type and mutant strains of *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. *Biological Control* 14: 1-6.
- Makowski, R.M. 1993. Effect of inoculum concentration, temperature, dew period, and plant growth stage on disease of round-leaved mallow and velvetleaf by *Colletotrichum gloeosporioides* f. sp. *malvae*. *Phytopathology* 83: 1229-1234.
- Makowski, R.M., and Mortensen, K. 1998. Latent infections and penetration of the bioherbicide agent *Colletotrichum gloeosporioides* f. sp. *malvae* in non-target field crops under controlled environmental conditions. *Mycological Research* 102: 1545-1552.
- Manion, P.D. 1981. *Tree Disease Concepts*. Prentice-Hall, Inc., Englewood Cliffs, NJ. 399p.
- Maor, R., Puyesky, M., Horwitz, B.A., and Sharon, A. 1998. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. *Mycological Research* 102: 491-496.
- Mark, W.R., Hawksworth, F.G., and Oshima, N. 1976. Resin disease: a new disease of lodgepole pine dwarf mistletoe. *Canadian Journal of Forest Research* 6: 415-424.
- Mathiasen, R.L. 1996. Dwarf mistletoes in forest canopies. *Northwest Science* 70 (Special Issue): 61-71.
- Mathre, D.E., Cook, R.J., and Callan, N.W. 1999. From discovery to use. Traversing the world of commercializing biocontrol agents for plant disease control. *Plant Disease* 83: 972-983.
- Mercer, P.C. 1988. Biological control of decay fungi in wood. *In* *Biocontrol of Plant Diseases*, Vol. I. *Edited by* Mukerji, K.G., and Garg, K.L. CRC Press Inc, Boca Raton, Florida. pp. 177-198.
- Mielke, J.L. 1959. Infection experiments with *Septogloeum gillii*, a fungus parasitic on dwarf mistletoe. *Journal of Forestry* 57: 25-26.
- Mondal, A.H., and Parbery, D.G. 1992. The spore matrix and germination in *Colletotrichum musae*. *Mycological Research* 96: 592-596.
- Mordue, J.E.M. 1971. *Glomerella cingulata*. C.M.I. Descriptions of Pathogenic Fungi and Bacteria. No. 315.
- Morin, L., Watson, A.K., and Reeleder, R.D. 1990. Effect of dew, inoculum density, and spray additives on infection of field bindweed by *Phomopsis convolvulus*. *Canadian Journal of Plant Pathology* 12: 48-56.

- Morin, L., Derby, J.L., and Kokko, E.G. 1996. Infection process of *Colletotrichum gloeosporioides* f. sp. *malvae* on Malvaceae weeds. *Mycological Research* 100: 165-172.
- Mortensen, K. 1986. Biological control of weeds with plant pathogens. *Canadian Journal of Plant Pathology* 8: 229-231.
- Mortensen, K. 1988. The potential of an endemic fungus, *Colletotrichum gloeosporioides*, for biological control of round-leaved mallow (*Malva pusilla*) and velvetleaf (*Abutilon theophrasti*). *Weed Science* 36: 473-478.
- Mortensen, K. 1998. Biological control of weeds using microorganisms. *In Plant-Microbe Interactions and Biological Control. Edited by G.J. Boland and L.D. Kuykendall.* Marcel Dekker, Inc. New York. pp. 223-247.
- Muir, J.A. 1967. Occurrence of *Colletotrichum gloeosporioides* on dwarf mistletoe (*Arceuthobium americanum*) in western Canada. *Plant Disease Reporter* 51: 798-799.
- Muir, J.A. 1970. Dwarf mistletoe spread in young lodgepole pine stands in relation to density of infection sources. *Bi-monthly Research Note* 26. Canada Department of Fisheries and Forestry, Forestry Service: 49.
- Muir, J.A. 1973. *Cylindrocarpon gillii*, a new combination for *Septogloeum gillii* on dwarf mistletoe. *Canadian Journal of Botany* 51: 1997-1998.
- Muir, J.A. 1977. Effects of the fungal hyperparasite *C. gloeosporioides* of dwarf mistletoe (*Arceuthobium americanum*) on young lodgepole pine. *Canadian Journal of Forest Research* 7: 579-583.
- Neumann, S., and Boland, G.J. 1999. Influence of selected adjuvants on disease severity by *Phoma herbarum* on dandelion (*Taraxacum officinale*). *Weed Technology* 13: 675-679.
- Nevill, R., and Winston, D. 1994. Forest health research needs survey. Forestry Canada. Pacific Forestry Centre. BCFRAC Report No. 3. FRDA Report 212.
- Nicholls, T. H. 1988. Ethephon tests for lodgepole pine dwarf mistletoe in Colorado. *Proceedings of the 36<sup>th</sup> Western International Forest Disease Work Conference*, Sept. 19-23, 1988, Park City, Utah, p. 34-37.
- Norman, D.J., and Trujillo, E.E. 1995. Development of *Colletotrichum gloeosporioides* f. sp. *Clidemiae* and *Septoria passiflorae* into two mycoherbicides with extended viability. *Plant Disease* 79: 1029-1032.

- Oleskevich, C., Shamoun, S.F., Vesonder, R.F., and Punja, Z.K. 1998. Evaluation of *Fusarium avenaceum* and other fungi for potential as biological control agents of invasive *Rubus* species in British Columbia. *Canadian Journal of Plant Pathology* 20: 12-18.
- Parker, A.K. 1970. Growth of *Wallrothiella arceuthobii* on artificial media. *Canadian Journal of Botany* 48: 837-838.
- Parks, C.G., Bull, E.L., Tinnin, R.O., Shepherd, J.F., and Blumton, A.K. 1999. Wildlife use of dwarf mistletoe brooms in Douglas-fir in northeast Oregon. *Western Journal of Applied Forestry* 14: 100-105.
- Parmeter, J.R. Jr. 1978. Forest stand dynamics and ecological factors in relation to dwarf mistletoe spread, impact and control. In: Scharpf, R.F., and Parmeter, J.R. Jr. tech. Cords. Proceedings of the symposium on dwarf mistletoe control through forest management, April 11 – 13, 1978, Berkeley, CA. General Technical Report PSW-31. Berkley, CA. U.S. Department of Agriculture, Forest Service, Pacific Southwest Forest and Range Experiment Station: 16-30.
- Parmeter, J.R. Jr., Hood, J.R., and Scharpf, R.F. 1959. *Colletotrichum* blight of dwarf mistletoe. *Phytopathology* 49: 812-815.
- Pearce, R.B. 1984. Staining fungal hyphae in wood. *Transactions of the British Mycological Society* 82: 564-567.
- Peterson, R.S. 1966. *Cronartium* mycelium parasitizing gymnosperm and angiosperm tissues simultaneously. *Mycologia* 58: 474-477.
- Piirto, D.D., Crews, D.L., and Troxell, H.E. 1974. The effects of dwarf mistletoe on the wood properties of lodgepole pine. *Wood and Fiber* 6: 26-35.
- Pitt, D.G., Dumas, M.T., Wall, R.E., Thompson, D.G., Lanteigne, L., Hintz, W., Sampson, G., and Wagner, R.G. 1999. *Chondrostereum purpureum* as a biological control agent in forest vegetation management. I. Efficacy on speckled alder, red maple, and aspen in eastern Canada. *Canadian Journal of Forest Research* 29: 841-851.
- Prusky, D., Freeman, S., and Dickman, M.B. eds. 2000. *Colletotrichum* Host Specificity, Pathology, and Host – Pathogen Interaction. APS Press, St. Paul, MN. 393p.
- Quimby, P.C., Zidack, N.K., Boyette, C.D., and Grey, W.E. 1999. A simple method for stabilizing and granulating fungi. *Biocontrol Science and Technology* 9: 5-8.
- Robinson, M., and Sharon, A. 1999. Transformation of the bioherbicide *Colletotrichum gloeosporioides* f. sp. *aeschynomene* by electroporation of germinated conidia.

Current Genetics 36: 98-104.

- Rose, C.L. 1996. Forest canopy – atmosphere interactions. Northwest Science 70 (Special Issue): 7-14.
- Roth, L.F. 1974. Resistance of ponderosa pine to dwarf mistletoe. *Silvae Genetica* 23: 116-120.
- Sands, D.C., Ford, E.J., and Miller, R.V. 1990. Genetic manipulation of broad host-range fungi for biological control of weeds. *Weed Technology* 4: 471-474.
- Scharpf, R.F. 1964. Cultural variation and pathogenicity of the *Colletotrichum* blight fungus of dwarf mistletoe. *Phytopathology* 54: 905-906.
- Schneider, H. 1981. Pathological and anatomy and mycology. *In* Staining Procedures, 4<sup>th</sup> ed. Edited by G. Clark. Williams and Wilkins, Baltimore. p. 372-373.
- Senaratna, L.K., Wijesundera, R.L.C., and Liyanage, A. de S. 1991. Morphological and physiological characters of two isolates of *Colletotrichum gloeosporioides* from rubber (*Hevea brasiliensis*). *Mycological Research* 95: 1085-1089.
- Sexton, A.G., and Howlett, B.J. 2001. Green fluorescent protein as a reporter in the *Brassica-Leptosphaeria maculans* interaction. *Physiological and Molecular Plant Pathology* 58: 13-21.
- Shamoun, S.F., Ramsfield, T.D., Shrimpton, G., and Hintz, W.E. 1996. Development of *Chondrostereum purpureum* as a mycoherbicide for red alder (*Alnus rubra*) in utility-rights-of-way. *In* Proceedings of the 1996 Expert Committee on weeds national meeting, Victoria, British Columbia, December 9 – 12, 1996. Edited by P. Comeau and G. Harper, B.C. Ministry of Forests, Research Branch, Victoria, B.C., Canada. p. 199.
- Shamoun, S.F., Countess, R.E., Vogelgsang, S., and Oleskevich, C. 2000. The mycobiota of salal (*Gaultheria shallon*) collected on Vancouver Island and the exploitation of fungal pathogens for biological control. *Canadian Journal of Plant Pathology* 22: 192.
- Shaw, C.G. III, and Loopstra, E.M. 1991. Development of dwarf mistletoe infections on inoculated western hemlock trees in southeast Alaska. *Northwest Science* 65: 48-52.
- Shen, S., Goodwin, P.H., and Hsiang, T. 2001. Infection of *Nicotiana* species by the anthracnose fungus, *Colletotrichum orbiculare*. *European Journal of Plant Pathology* 107: 767-773.
- Smith, R.B. 1977. Overstory spread and intensification of hemlock dwarf mistletoe.

- Smith, R.B., and Wass, E.F. 1979. Infection trials with three dwarf mistletoe species within and beyond their known ranges in British Columbia. *Canadian Journal of Plant Pathology* 1: 47-57.
- Smith, R.B., Wass, E.F., and Meagher, M.D. 1993. Evidence of resistance to hemlock dwarf mistletoe (*Arceuthobium tsugense*) in western hemlock (*Tsuga heterophylla*) clones. *European Journal of Forest Pathology* 23: 163-170.
- Sproule, A. 1996. Branch age in jack pine at the time of dwarf mistletoe infection. *The Forestry Chronicle* 72: 307.
- Srivastava, L.M., and Essau, K. 1961. Relation of dwarf mistletoe (*Arceuthobium*) to the xylem tissue of conifers. I. Anatomy of parasite sinkers and their connection with host xylem. *American Journal of Botany* 48: 159-167.
- Steen, O.A., and Coupé, R.A. 1997. Field guide to forest site identification and interpretation for the Cariboo Forest Region. British Columbia Ministry of Forests, Victoria, B.C., Land Management Handbook 39.
- Stojanović, S. 1989. Proučavanje *Sphaeropsis visci* (Sallm) Sacc. I *Colletotrichum gloeosporioides* (Sacc.) Penz. – parazita bele imele. *Zaštita bilja* 40 (4), br. 190: 493-503.
- Sutton, B.C. 1980. The Coelomycetes. Fungi Imperfecti with Pycnidia, Acervuli and Stromata. Commonwealth Mycological Institute, Kew, UK. 696p.
- Templeton, G.E. 1982. Biological herbicides: Discover, development, deployment. *Weed Science* 30: 430-433.
- Templeton, G.E. 1992. Use of *Colletotrichum* strains as mycoherbicides. In *Colletotrichum* Biology, Pathology and Control. Edited by J.A. Bailey and M.J. Jeger. C.A.B. International. Wallingford, Oxon, UK. p. 358-380.
- Trujillo, E.E., Norman, D.J., and Killgore, E.M. 1994. Septoria leaf spot, a potential biological control for banana poka vine in forests of Hawaii. *Plant Disease* 78: 883-885.
- van der Kamp, B.J., and Blenis, P.V. 1996. A model of hyperparasite regulation of the gall rust – lodgepole pine pathosystem. *Canadian Journal of Forest Research* 26: 1256-1265.
- Van Sickle, G.A., and Wegwitz, E. 1978. Silvicultural control of dwarf mistletoe in young lodgepole pine stands in Alberta and British Columbia. Environment Canada. Forestry Service. Pacific Forest Research Centre Information Report

BC-X-180. 11p.

- Wall, R.E. 1991. Pathological effects of *Chondrostereum purpureum* in inoculated yellow birch and beech. *Canadian Journal of Plant Pathology* 13: 81-87.
- Walker, H.L., and Connick, W.J. Jr. 1983. Sodium alginate for production and formulation of mycoherbicides. *Weed Science* 31: 333-338.
- Wastie, R.L. 1972. Secondary leaf fall of *Hevea brasiliensis*: meteorological and other factors affecting infection by *Colletotrichum gloeosporioides*. *Annals of Applied Biology* 72: 283-293.
- Watson, A.K., and Wall, R.E. 1995. Mycoherbicides: Their role in vegetation management in Canadian forests. *In* Recent progress in forest biotechnology in Canada, *Edited by* Charest, P.J., and Duchesne, L.C. Petawawa National Forestry Institute, Canadian Forest Service, Information Report PI-X-120. pp. 74-82.
- Watson, A.K., Gressel, J., Sharon, A., and Dinoor, A. 2000. *Colletotrichum* strains for weed control. *In* *Colletotrichum* host specificity, pathology and host-pathogen interaction. *Edited by* D. Prusky, S. Freeman, M.B. Dickman. APS Press, St. Paul, Minnesota. pp. 245-265.
- Wicker, E.F. 1967. Appraisal of biological control of *Arceuthobium campylopodum* f. *campylopodum* by *Colletotrichum gloeosporioides*. *Plant Disease Reporter* 51: 311-313.
- Wicker, E.F., and Shaw, C.G. 1968. Fungal parasites of dwarf mistletoes. *Mycologia* 60: 372-383.
- Weir, J.R. 1915. *Wallrothiella arceuthobii*. *Journal of Agricultural Research* 4: 369-378.
- Wilcox, W.W., Pong, W.Y., and Parmeter, J.R. 1973. Effects of mistletoe and other defects on lumber quality in white fir. *Wood and Fiber* 4: 272-277.
- Wilson, C.L. 1969. Use of plant pathogens in weed control. *Annual Review of Phytopathology* 7: 411-434.
- Winder, R.S., and Watson, A.K. 1994. A potential microbial control for fireweed (*Epilobium angustifolium*). *Phytoprotection* 75: 19-33.
- Wood, C. 1986. Distribution maps of common tree diseases in British Columbia. Pacific Forestry Centre, Victoria, BC. Information Report BC-X-281. 68p.
- Yang, X., Madden, L.V., Wilson, L.L., and Ellis, M.A. 1990. Effects of surface topography and rain intensity on splash dispersal of *Colletotrichum acutatum*.

Phytopathology 80: 1115-1120.

**Appendix 1 – Temperature on inoculation day (July 23, 2000) and immediately following.**

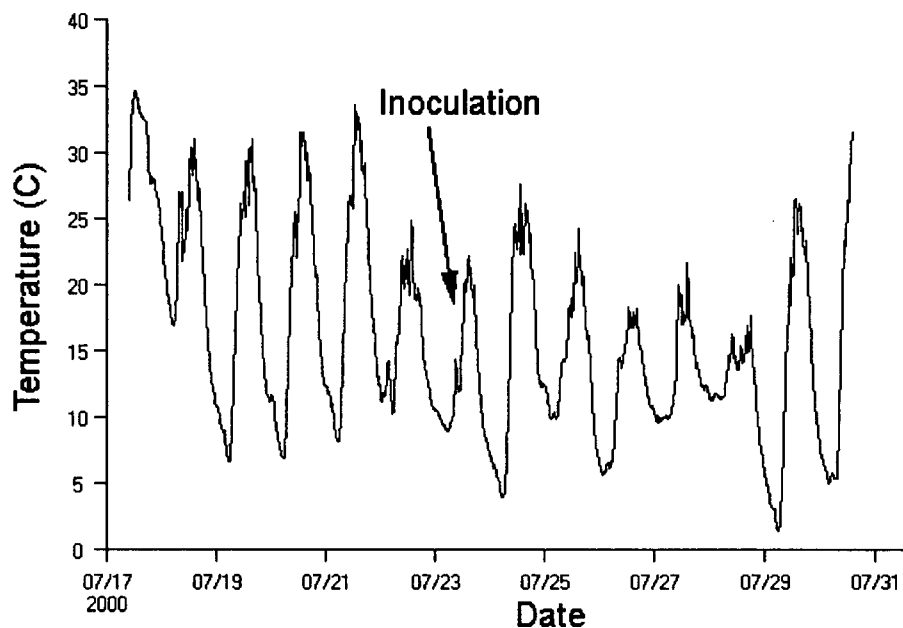


Figure appendix 1-1. Temperature on inoculation day and following. Near Lytton, British Columbia. Maximum temperature on inoculation day was 22°C at 3:19 PM.

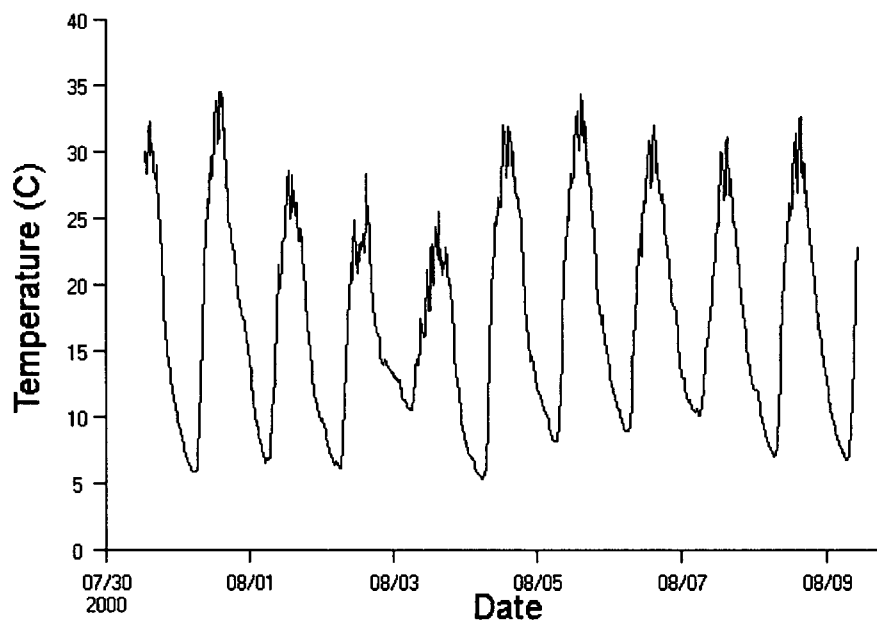


Figure appendix 1-2. Temperature at inoculation site from July 30 to August 9, 2001. Near Lytton, British Columbia.

## Appendix 2 – Relative humidity on inoculation day and immediately following

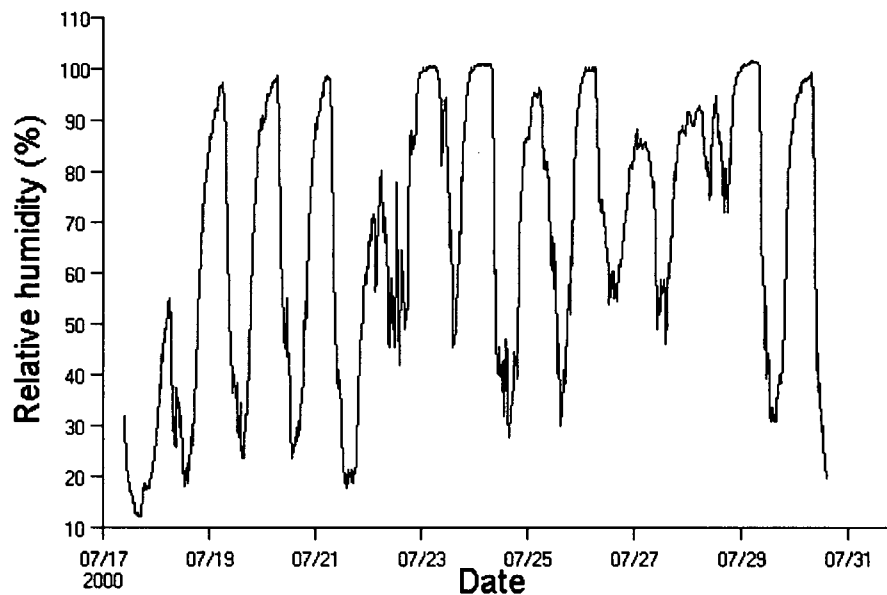


Figure appendix 2-1. Relative humidity on inoculation day and immediately following. Minimum relative humidity at inoculation was 46.6% at 3:19 PM. Near Lytton, British Columbia.

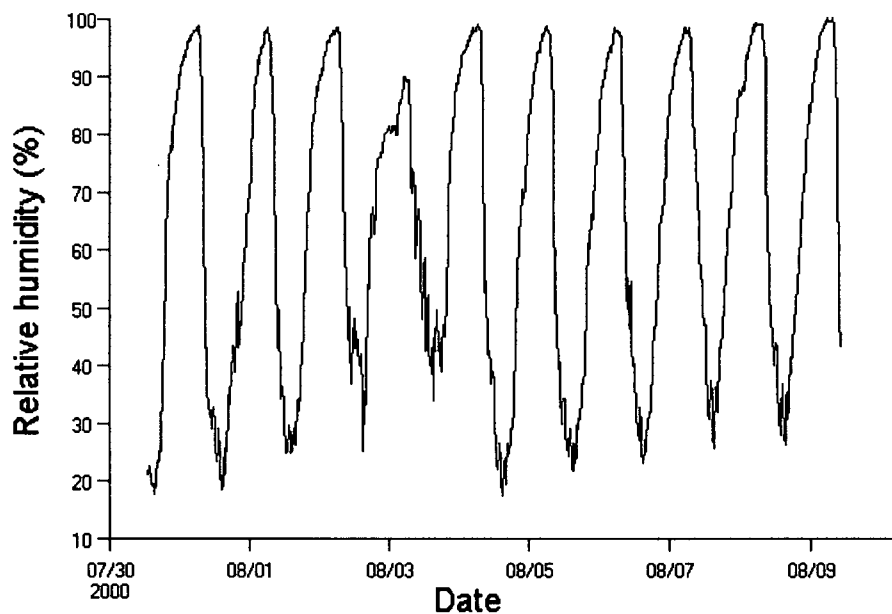


Figure appendix 2-2. Relative humidity at inoculation site from July 30 to August 9, 2001. Near Lytton, British Columbia.

### Appendix 3 – Histological staining methods

Safranin – Picro=Aniline blue (Schneider, 1981)

Hemo de (FisherBrand, Pittsburgh, PA)	15 min
Hemo de	5 min
Absolute ethanol / Hemo de	2 min
Absolute ethanol	2 min
Celloidin	Dip
95% ethanol	2 min
70% ethanol	2 min
50% ethanol	2 min
30% ethanol	2 min
18% ethanol	2 min
Double distilled H <sub>2</sub> O	5 min
1% Safranin	1 min
Tap H <sub>2</sub> O	1 + min
Picro+Aniline Blue	Simmer
18% ethanol	2 min
30% ethanol	2 min
50% ethanol	2 min
70% ethanol	2 min
95% ethanol	2 min
Absolute ethanol	2 min
Absolute ethanol / Hemo de	2 min
Clearing Solution <sup>†</sup>	5 min
Hemo de	5 min
Hemo de	15 min
Permout and cover slip	

<sup>†</sup>Clearing solution = 50% clove oil, 25% absolute ethanol, 25% xylene

Johansen's Safranin (Johansen, 1940)

Hemo de	15 min
Hemo de	5 min
Absolute ethanol / Hemo de	2 min
Absolute ethanol	2 min
Celloidin	Dip
95% ethanol	2 min
70% ethanol	2 min
50% ethanol	2 min
Johansen's Safranin in 95% ethanol	20 min
50% ethanol with trace HCl	10 sec
Picro=Analine Blue in 95% ethanol	20 min
100% ethanol	10 sec
Clearing solution	5 min
Hemo de	5 min
Hemo de	15 min
Permout and cover slip	

# Rhodamine B – Methyl Green (Modified from Pearce, 1984)

Hemo de	15 min
Hemo de	5 min
Absolute ethanol / Hemo de	2 min
Absolute ethanol	2 min
Celloidin	Dip
95% ethanol	2 min
70% ethanol	2 min
50% ethanol	2 min
30% ethanol	2 min
18% ethanol	2 min
Double distilled H <sub>2</sub> O	5 min
1% Rhodamine-B	20 min
Tap H <sub>2</sub> O	Rinse
0.15% Methyl Green in 0.2M	5 min
Phosphate Buffer pH 8.0	
Destain <sup>‡</sup>	5 min
Clearing Solution	5 min
Hemo de	5 min
Hemo de	15 min
Permout and cover slip	

<sup>‡</sup>Destain: 40% d H<sub>2</sub>O, 50% methanol, 10% acetic acid

#### **Appendix 4 – ANOVA tables for canopy study, Chapter 6**

Table appendix 4-1. Two-way analysis of variance to compare trees and canopy thirds.

Source	DF	SS	MS	F	P
Tree	5	548.206	109.641	0.233	0.939
Canopy third	2	882.243	441.122	0.938	0.423
Residual	10	4701.338	470.134		
Total	17	6131.787	360.693		

Table appendix 4-2. Two-way analysis of variance to compare trees and canopy aspect.

Source	DF	SS	MS	F	P
Tree	5	728.977	145.795	0.763	0.591
Canopy aspect	3	97.945	32.648	0.171	0.914
Residual	15	2867.849	191.190		
Total	23	3694.771	160.642		

## Appendix 5 – Diagrammatic representation of the lifecycle of *Caliciopsis arceuthobii* and fruit production by *Arceuthobium* *americanum*

The diagrams below represent the lifecycle of *C. arceuthobii* and *A. americanum* and can be utilized to aid in the understanding of how individual cohorts of *C. arceuthobii* perithecia and *A. americanum* fruit are related in time. Data collection represented by an “A”. *Caliciopsis* perithecia are visible all year. The *C. arceuthobii* diagram was constructed based on the life cycle information in Kuijt (1969) and the *A. americanum* diagram was based on information presented in Hawksworth and Wiens (1996). A complete circuit around the diagram represents one year.

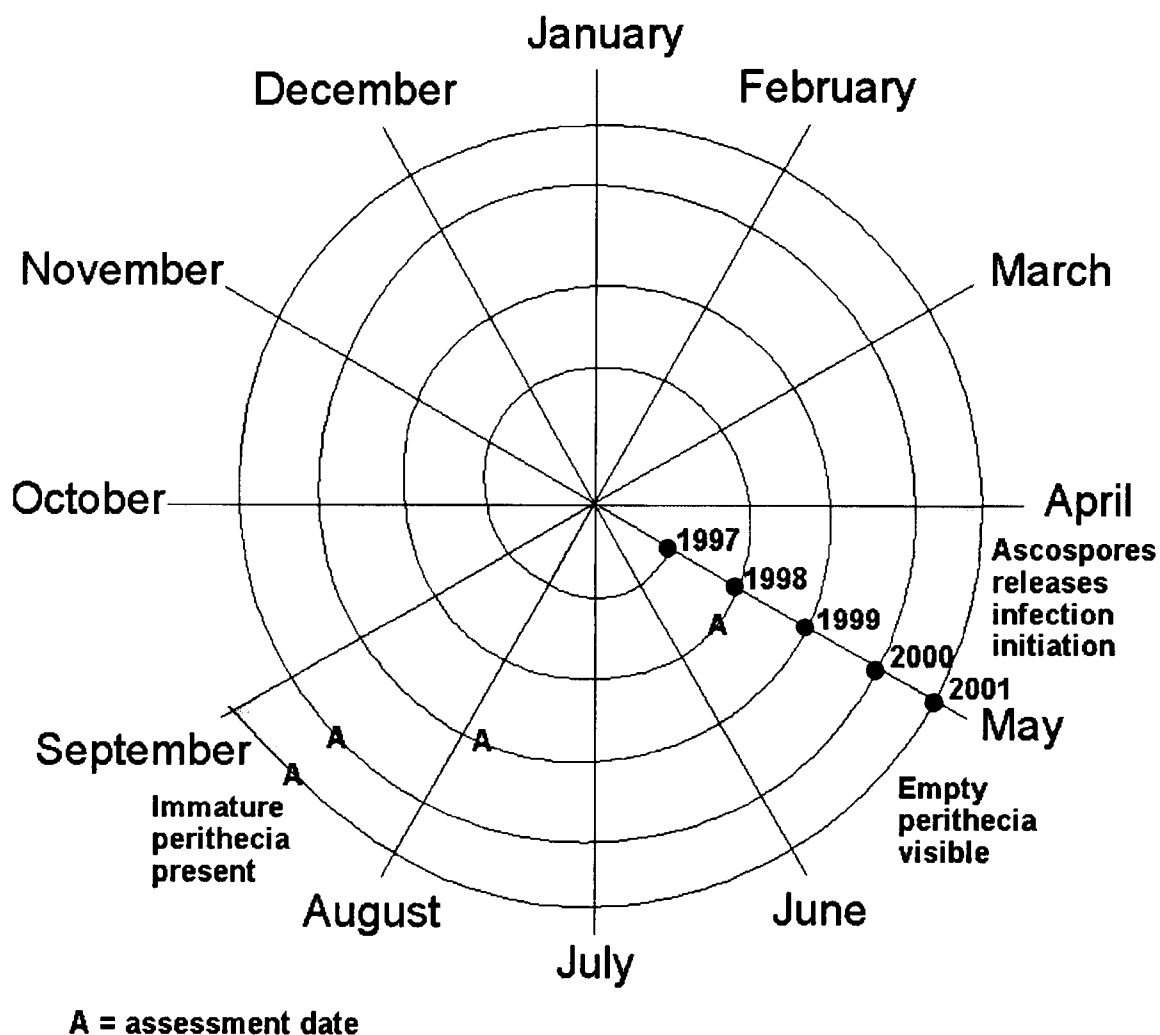


Figure appendix 5-1. Timing of ascospore release and new perithecia production by *Caliciopsis arceuthobii* infecting *Arceuthobium americanum*.

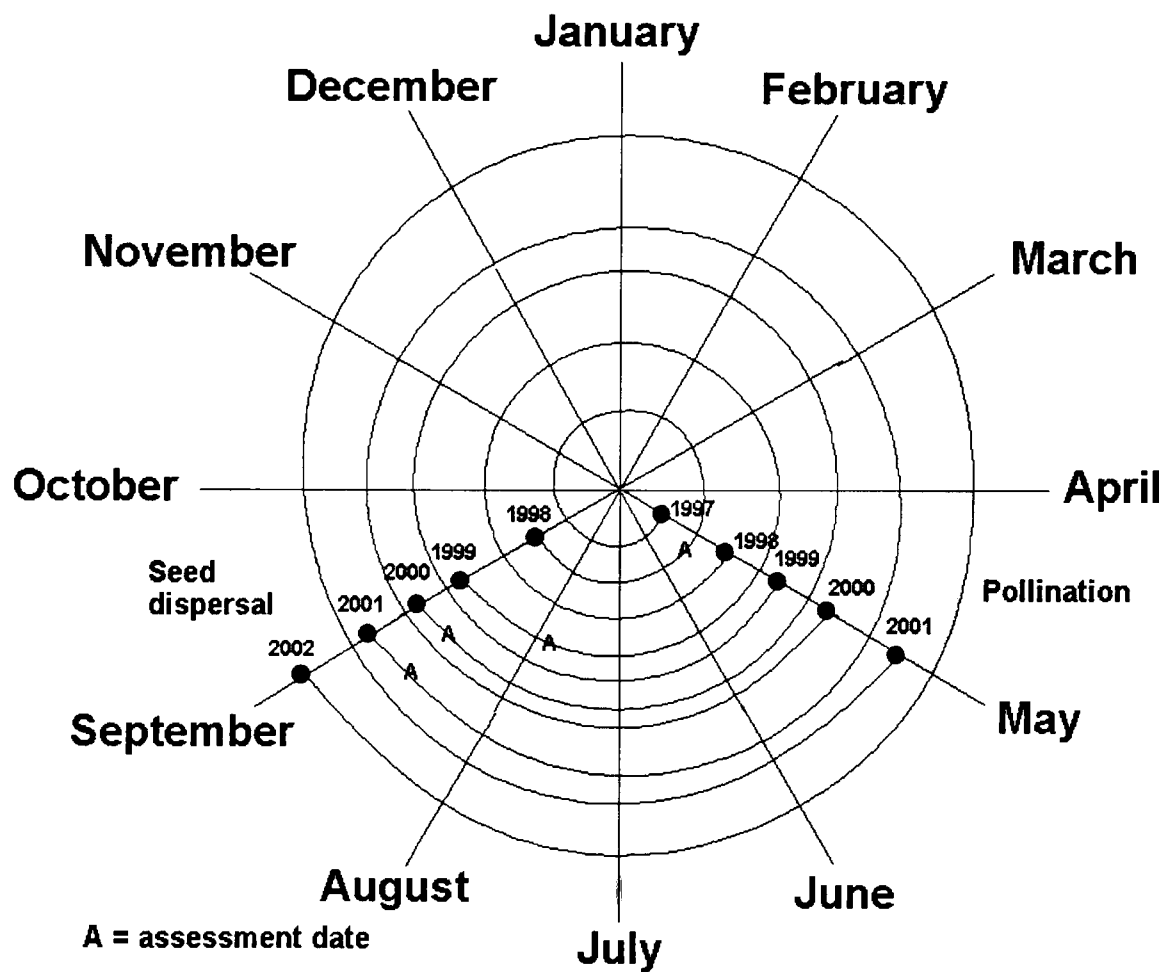


Figure appendix 5-2. Fruit production on *Arceuthobium americanum*.

## Appendix 6 – Presence of *Caliciopsis arceuthobii* on *Arceuthobium americanum*

Table appendix 6-1. Number of *Caliciopsis arceuthobii* infected *Arceuthobium americanum* flowers observed at the Knife Creek Block of the Alex Fraser Research Forest, near 150 Mile House, British Columbia.

Replicate	Number of flowers with <i>C. arceuthobii</i> perithecia			
	1998	1999	2000	2001
1	8	0	0	17
2	5	35	35	3
3	17	5	0	2
4	1	0	0	4
5	32	0	0	2
6	0	0	0	Dead
7	0	0	0	1
8	0	39	0	0
9	4	Dead	Dead	Dead
10	0	0	Dead	Dead
11	0	0	0	0
12	180	103	149	0
13	27	30	11	27
14	125	Dead	Dead	Dead
15	36	Dead	Dead	Dead
16	39	206	22	23
17	400	0	0	44
18	80	Dead	Dead	Dead
19	169	0	0*	0
20	250	63	4	5
21	0	Dead	Dead	Dead

Replicate	Number of flowers with <i>C. arceuthobii</i> perithecia			
	1998	1999	2000	2001
22	150	0	63	111
23	0	2	8	Dead
24	28	1	56	12
25	154	Dead	Dead	Dead
26	300	56	423	73
27	100	12	0	3
28	51	168	0	2
29	87	Dead	Dead	Dead
30	0	0	0*	0*

\* Indicates that there were no shoots present on the *A. americanum* infection. These replicates were not included in the Fisher exact test calculations.