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Development of a *Colletotrichum dematium* as a bioherbicide for the control of fireweed

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Short title:

A *Colletotrichum dematium* as a fireweed bioherbicide

Christian Léger

Abstract

An anthracnose-inducing pathogen, *Colletotrichum dematium*, was studied as a bioherbicide for *Epilobium angustifolium*. A comparative study involving other *C. dematium* isolates suggests that the isolate from *E. angustifolium* is a *forma specialis* and should be designated as *Colletotrichum dematium* f.sp. *epilobii*. Under controlled environment conditions, the most severe damage was achieved on seedlings using a conidial density of $1x10^9$ conidia m^{-2}. Virulence decreased with plant maturity. Satisfactory levels of control were limited to long dew duration (> 18 h) and high temperature treatments. Of various adjuvants tested, significantly higher levels of control were achieved when inoculum was sprayed in a vegetable oil emulsion (25 % v/v) ($P < 0.05$). An inoculum buffered to acidic pH levels (pH 3.0) similarly increased level of control compared with an unbuffered conidial suspension and the adjustment to more alkaline pH levels using the citrate-phosphate buffer (pH > 4.0), inhibited disease expression. In host range studies, *C. dematium* f.sp. *epilobii* was restricted to the Onagraceae family. Both *Camissonia bistorta* and *Clarkia pulchella* were susceptible whereas the fungus was highly virulent on all but one *E. angustifolium* ecotype. Among nine commercial tree species tested for their susceptibility to *C. dematium* f.sp. *epilobii*, yellow birch (*Betula alleghaniensis*) was susceptible to the fungus when conidia were applied in an oil emulsion. Under field conditions, the application of inoculum in a tank mix combination with the oil emulsion and a low rate of glyphosate provided significant growth control of *E. angustifolium* seedlings (7-wk-old), whereas the effectiveness of this formulated conidial suspension significantly decreased with plant maturity. Under controlled conditions, post-emergence application of an oil-based formulation including the ground colonized substrate of another bioherbicide candidate of *E. angustifolium*, *Alternaria* sp., significantly reduced above-ground biomass when provided a 12-h dew and applied at a rate as low as $5x10^6$ conidia m^{-2}.

Résumé

Un champignon phytopathogène, *Colletotrichum dematium*, a été étudié comme bioherbicide de *Epilobium angustifolium*. Une étude comparative menée avec d'autres isolats de la même espèce suggère que celui provenant de *Epilobium angustifolium* est un *forma specialis* et devrait par ce fait être désigné comme *Colletotrichum dematium* f.sp. *epilobii*. Sous conditions contrôlées, les dommages les plus sévères ont été obtenus suivant l'application du champignon à une densité de $1x10^9$ conidies m^{-2} sur des plantules. La virulence fut considérablement réduite chez les plants plus âgés. L'efficacité fut d'autre part limitée à des niveaux élevés de températures et de durées de rosée (> 18 h). Le niveau de contrôle a été significativement amplifié par l'addition d'une émulsion à base d'huile végétable (25% v/v) ($P < 0.05$). Similairement, l'ajustement du pH de l'inoculum (pH 3.0) a augmenté le niveau de contrôle comparativement à un inoculum non-tamponé, alors que l'ajustement à pH plus alkalins à l'aide du tampon citrate-phosphate a inhibé le développement de la maladie. Le spectre d'activité de *C. dematium* f.sp. *epilobii* s'est taxonomiquement limité à la famille Onagraceae. Les espèces *Camissonia bistorta* et *Clarkia pulchella* ainsi que quatre des cinq écotypes de *E. angustifolium* étudiés se sont démontrés susceptibles à *C. dematium* f.sp. *epilobii*. Le champignon fut pathogène envers le bouleau jaune lorsque les conidies furent appliquées avec l'émulsion d'huile. Dans les essais au champ, l'efficacité d'un inoculum formulé avec l'émulsion d'huile et une faible dose de glyphosate a significativement varié selon l'âge de la plante. Sous conditions contrôlées, l'application conjointe d'une émulsion d'huile végétale et du substrat de croissance d'un autre candidat de lutte biologique de *E. angustifolium*, *Alternaria* sp., a significativement réduit la biomasse des plantules avec une durée de rosée de 12 h et une densité d'application de $5x10^6$ conidies m^{-2}.

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Description of Thesis Format

This thesis is comprised in the form of original papers suitable for journal publication. The first chapter is a general introduction presenting the background knowledge and objectives of the research. Chapters 2, 3, 4, 5, and 6 constitute the body of the thesis in which each chapter are a complete manuscript and linked with connecting texts so as to establish logical bridges between the different papers. A general discussion and conclusion are included in chapter 7. The last section is presented as an appendix and includes results from experiments not discussed in previous chapters. Part of the thesis was presented and published as an abstract at the 63rd ACFAS Congress, held in Chicoutimi, Québec, in 1995.

This thesis format is in accordance with conditions outlined in part B, section 2 of the "Guidelines Concerning Thesis Preparation" from the Faculty of Graduate Studies and Research, McGill University, which read as follows:

"*Candidates have the option, subjected to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data as well as descriptions of equipment*

used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of whom contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

Manuscripts from chapters 2, 3, 4, and 5 are co-authored by both Dr. Steve G. Hallett and Dr. Alan K. Watson, whereas the manuscript from chapter 6 is co-authored by Dr. A.K. Watson. The candidate (Christian Léger) performed all the experimental research, statistical analyses, and is the primary author of all five manuscripts. Both Drs. Steve G. Hallett and Alan K. Watson provided supervisory guidance and assisted in manuscript preparation.

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Chapter 1

General introduction

1.1. Undesirable vegetation in forestry.

The boreal forest is mainly composed of conifer species and covers about one third of the Canadian territory. The availability and low cost of this timber resource has provided Canada with a competitive advantage for the past 25 years, thus making it one of the world's leading sources of wood products (Schuler and Meil, 1990). However, this status was obtained by employing silvicultural practices that were largely based on a view of an overabundance and inexhaustible wood resource. By the beginning of the 1980's however, some difficulties in wood fiber supply were recognized. For example, in Québec, enhanced wood exploitation created by industrial expansion as well as wood fiber losses caused by the spruce budworm, had created an unbalance between wood fiber availability and demand (Gouvernement du Québec, 1991). It was then apparent that forests would not be able, in the short-term, to satisfy the projected increases in demand for wood products and other forest resources (Schuler and Meil, 1990). In order to maintain Canada's competitive advantage in wood production, forest managers must reevaluate fully conventional and non-sustainable forest management practices as well as provide alternate silvicultural management strategies that ensure the appropriate control of competing vegetation.

As in agricultural systems, the control of undesirable vegetation constitutes an important aspect of silvicultural activities. Appropriate control measures lead to increased productivity, both in yield and in time, by decreasing the degree of competition and reducing the time interval between harvesting sequences.

According to Klingman (1966), weeds are undesirable plants growing in situations where they are not wanted, or simply plants out of place. This definition has evolved primarily from agricultural systems, and in this respect, weeds have been traditionally perceived from a competitive point of view. However, considering the numerous activities that are performed during stand development, the concept of a weed encompasses a broader spectrum in forestry. It not only includes plant species that reduce growth of desireable species but also those species wich interfere with silvicultural operations (Walstad and Kuch, 1987). These species generally differ from those that affect agriculture as most are native perennial (Watson and Wall, 1995). Hence, in forestry, weeds are commonly defined as undesirable vegetation. For example, the suppression of species to facilitate access to planting sites and the cutting of hardwood species at the time of pre-commercial thinning of a coniferous stand constitute two important vegetation management practices.

However, vegetation management practices are mainly performed during the early phase of forest regeneration, where activities associated with harvesting practices, such as soil disturbance and canopy clearing, provide a favourable environment for weed emergence. Consequently, the concept of undesirable vegetation is mostly associated with plant species known to have deleterious effects on the survival and growth of commercial

species (Bell, 1991). For example, allelopathy (Fisher, 1980), the creation of favourable habitats for animals, the physical damage due to smothering by dead foliage, and fire hazards associated with dry dead vegetation (Estabrooks, 1988), constitute different ways by which vegetation can affect the survival and growth of newly planted trees. However, control practices are generally performed with a view to reducing the degree of competition from associated vegetation. In the absence of an adequate supply of light, moisture, nutrients, and space, it is clear that commercial tree seedlings may die or, at best, grow at a rate well below their physiological optimum (Bell, 1991).

1.2. Weed management in forestry: a practice in transition.

A number of silvicultural treatments can be used to reduce non-commercial competing vegetation. For example, mechanical control and prescribed burning can be used effectively in specific situations, particularly in areas where herbicide use is restricted. However, such vegetation control methods are limited by cost, slope, and predisposition of the site to soil erosion (Watson and Wall, 1995), and specifically for prescribed burning, conditions must be suitable, both from the stand point of safety (risk of uncontrolled fire) as well as efficacy (suitability of weather conditions). Furthermore, these practices may actually improve conditions for establishment of some non-crop species. For instance, many herbaceous and hardwood species are capable of rapidly colonizing newly burned sites, thereby hampering conifer regeneration (Walstad *et al.*, 1987).

Due to their low-cost and versatility of application, both in time and space, vegetation management practices in Canada have focused mainly on the utilization of

chemical herbicides (Campbell, 1990). In some cases (i.e. stand release), herbicides constitute the only practical and suitable way of controlling undesirable vegetation (Walstad *et al.*, 1987). As reported by Campbell (1990), 0.14% of productive forest land, 18% of the harvest area, and 71% of planted area were treated with chemical herbicides in Canada during 1988. About 85% of the herbicides used in Canadian forests are for stand tending, and 15% for site preparation.

Nine herbicides are registered for forest vegetation management in Canada and three of these are used in Québec: glyphosate (*N*-[phophonomethyl]glycine), hexazinone (3-cyclohexyl-6-[dimethylamino]-1-methyl-1,3,5-triazine-2,4[1*H*,3*H*]-dione), and simazine (6-chloro-*N*,*N*'-diethyl-1,3,5-triazine-2,4-diamine) (Gouvernement du Québec, 1991).

Although the benefits of chemical control have long been recognized, several factors provide impetus for developing alternative or complementary control strategies. The potential adverse effects of chemical herbicides on young conifer seedlings are well known (Boyd *et al.*, 1985; Chapeskie *et al.*, 1989). Moreover, in conjunction with demands to enhance forest management, there is increasing pressure in North America to reduce herbicide use (Walstad, 1988), and in Québec, a total ban on chemical herbicides in the forest sector is projected by the year 2001. This ban constitutes one of the foremost objectives of the new Québec forest management program (Gouvernement du Québec, 1991).

Biological control, especially the mycoherbicide approach, may help reduce dependence on chemical herbicides for managing undesirable forest vegetation. In Canada, a national research network, BICOVER, involving research groups from both government

research institutes and universities was formed in 1990 with the goal of focusing research efforts in the discovery and development of biological and/or biorational control strategies as silvicultural practices for the control of undesirable vegetation (Thompson *et al.*, 1992).

1.3. Biological weed control: general principles.

Biological control of weeds is the deliberate use of natural enemies (exotic or native) to control weed populations to a tolerable level. This method has been primarily used in agriculture, with records of such use going back more than two centuries. As reported by Schroeder (1983), the first intentional introduction of a natural enemy for weed control occurred in 1795, when the cochineal insect, *Dactylopius ceylonensis* Green was introduced into India from Brazil in an attempt to control the widespread infestation of prickly pear (*Opuntia vulgaris* Miller). Since then, 180 species have been targeted worldwide for biocontrol (Julien, 1992).

There are two main approaches to the biological control of weeds: (1) the inoculative approach (classical) and (2) the inundative approach (bioherbicide or mycoherbicide). The classical method involves the introduction of biocontrol organisms (usually an insect) capable of causing self-sustaining epidemics following inoculative releases into a susceptible weed population (Charudattan, 1990). Inoculative agents are used against exotic weeds present in relatively unmanaged, undisturbed areas, where they have greater chance to become established compared with disturbed habitats (Andres, 1977; Charudattan, 1990). Perhaps the most successful and most often reported weed biocontrol project using the classical approach is the Australian introduction of the

Cactoblastis moth (*Cactoblastis cactorum* Berg.) for the control of *Opuntia* spp. In addition, the successful introduction of a rust fungus, *Puccinia chondrillina* Bubak & Sydenham, from Mediterranean regions to control rush skeletonweed (*Chondrilla juncea* L.) in Australia and the United States constitutes an example of the classical strategy using a fungus (Schroeder, 1983).

The inundative approach generally refers to the mass application of an indigenous pathogen, for the control of a specific weed species and is based on the fundamental principles of plant disease epidemiology. For practical production reasons, this method relies generally on the utilization of a fungus as the biocontrol agent, and the technique is therefore commonly called the bioherbicide or mycoherbicide approach. This method is relatively new compared with the classical approach, but the registration of three fungi in North America has demonstrated its potential. Bioherbicides are pathogens that have co-evolved with their weed hosts in particular regions resulting in homeostatic relationships characteristic of endemic diseases. As reported by Charudattan (1990), normally, they do not produce self-sustaining epidemics of destructive potential sufficient to afford weed control. However, by applying inundative doses of inoculum, they can be used to create a temporary, but highly destructive, level of disease. The control agents are cultured and mass produced in an artificial substrate, harvested, prepared to withstand storage and handling, and applied in a manner similar to chemical herbicides. Therefore, application can be carried out at the best time, when the target weed is at a susceptible stage and when environmental conditions are favourable (Charudattan, 1991).

Bioherbicide research is commonly performed in three main steps: discovery, development, and deployment (Templeton, 1982). The discovery phase implies literature and herbarium searches of previously reported diseases on the target weed as well as the collection of diseased plant material. The discovery phase is subsequently accomplished by the isolation, demonstration of Koch's postulates, identification, and the storage of the causal organism. As proposed by Daniel *et al.* (1973), potential bioherbicide pathogens must (a) be able to produce abundant and viable inoculum in artificial culture, (b) be genetically stable and specific for the target weed, and (c) be able to infect and kill the weed in environments of reasonably wide latitude. The development phase evaluates the potential of the candidate bioherbicide. Studies are conducted under controlled conditions in order to determine optimal conditions for disease expression while the degree of specificity is assessed through host range testing. The deployment stage involves the large-scale testing of the potential bioherbicide on the target weed under field conditions, and includes registration, large-scale production, marketing, and commercialization of the bioherbicide.

As stated by Charudattan (1990), several biological, technological, and economic constraints may restrict the development and practical use of bioherbicides. For instance, moisture and temperature conditions existing under field conditions are often insufficient in meeting the environmental requirements of the bioherbicide candidate for spore germination and host penetration. A biocontrol formulation constitutes a means by which this problem may be overcome, a way that may give effective and consistent weed control over a range of environmental conditions (Connick *et al.*, 1989). Formulation is the

blending of active ingredients (fungal propagules) with appropriate adjuvants and carriers in order to alter the physical characteristics of the inoculum to a more desirable form (Boyette *et al.*, 1991). Various adjuvants, such as sucrose (Walker, 1981), gelatin (Ormeno-Nunez *et al.*, 1988) or Sorbo™ (64% sorbitol, Atkemix Inc., Branford, ON) (Wymore and Watson, 1986) have been used successfully to either improve or modify spore germination, pathogen virulence, or environmental requirements. By substantially decreasing the amount of dew required for spore germination and infection of the target weed, research using an invert (water-in-oil) emulsion consisting of paraffin wax, mineral oil, lecithin, and water has also shown potential to overcome some of these constraints (Quimby *et al.*, 1989).

Three bioherbicides have been registered for commercial use in North America. Devine®, a liquid formulation of chlamydospores of a pathotype of *Phytophtora palmivora* (Butler) Butler was registered in 1981 for the control of stanglervine (*Morrenia odorata* [H. & L.] Lindl), a weed of citrus in Florida (Kenney, 1986; Charudattan, 1990). Collego®, a dry formulation of *Colletotrichum gloeosporioides* (Penz.) Sacc. f.sp. *aeschynomene*, an anthracnose-inciting pathogen, was registered in 1982 and successfully deployed in rice and soybean crops for the control of northern joinvetch (*Aeschynomene virginica* L.) (Charudattan, 1990). BioMal®, a spore formulation of *C. gloeosporioides* f.sp. *malvae*, for the control of round-leaved mallow (*Malva pusilla*), is registered in Canada (Makowski and Mortensen, 1992).

Although application of this approach to date has been largely confined to agriculture, several fungi have been investigated, and could be registered in the near future

in the forestry sector. Prospective advantages of the use of natural biological agents include (1) selective control of particular target weed species; (2) energy efficiency; and (3) comparatively inexpensive once developed (Walstad *et al.*, 1987). In pine plantations in Minnesota, *Ceratocystis fagacearum* (Bretz) Hunt was used with success in the 1950's as a biocontrol agent to control undesirable oak (*Quercus* spp.) (French and Schroeder, 1969). The causal agent of persimmon wilt, *Cephalosporium diospyri* Crandall, was used to prevent stump sprouting of common persimmon (*Diospyros virginiana* L.), a problematic weed species in pastures in the eastern and southeastern United States (Wilson, 1965). In Canada, several fungi are being investigated as potential silvicide agents (Watson and Wall, 1995). For instance, the potential of a formulation of *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar to control resprouting of cut deciduous competing species was recently demonstrated, and its large-scale use in forestry is under consideration (Wall, 1990; Gosselin and Jobidon, 1995).

1.4. *Epilobium angustifolium* biology and control.

Epilobium angustifolium L. ([*Chamaenerion angustifolium* (L.) Scop.] fireweed, rosebay, willow-herb, bouquets rouges, épilobe à feuilles étroites (Marie-Victorin, 1964; Scoggan, 1978; Broderick, 1990)) is native to North America and distributed throughout Canada. In forestry, the plant is an aggressive invader of recently logged sites (Dyrness, 1973) and can be an important competitor of coniferous trees within the first two years of site preparation (Haeussler and Coates, 1986), either by reducing the quality of sunlight reaching small conifer seedlings and/or the quantity of carbon dioxide assimilation of

conifer foliage (Comeau, 1988). In prolific *E. angustifolium* stands with other pioneers, seedling growth of commercially important tree species such as Douglas fir (*Pseudotsuga menziensii* (Mirb.)) can be suppressed (Peterson and Newton, 1983). *E. angustifolium* is also known to be an alternate host to the rust, *Pucciniastrum Abieti-chamenerii* Kleb. (=*Pucciniastrum epilobii* Oth.) that causes needle rust in *Abies balsamea* L. (Goodwin, 1930). In the Québec forest sector, is recognized as one of the four major weeds (Gouvernement du Québec, 1991), and is included in the noxious weed act of Manitoba (Broderick, 1990).

E. angustifolium belongs to the Onagraceae family. It is a perennial herb with erect, leafy stems that may exceed 2 m in height that arise each spring from buds formed the previous year on lateral roots (Broderick, 1990). The leaves are alternate, entire, rarely more than 15 cm long and 3 cm wide, green above, pale and reticulate-veiny beneath, acuminate with a narrowed, sessile to obscurely petiolate base (Scoggan, 1978; Broderick, 1990). More than 15 flowers are commonly borne on elongated, bracted racemes. Flowers are often magenta to pink. The style is pubescent and declined at the base, and the slender 3-4 mm long stigmas are revolute at maturity. Small light brown seeds are in canescent seed capsules, 2.5 - 8.0 cm in length. Seeds (0.8 - 1.3 mm in length) are capped with a tuft of hairs up to 13 mm long (Broderick, 1990). In North America, *E. angustifolium* occurs as two chromosomally different subspecies: a more northerly subspecies, ssp. *angustifolium* Mosquin (n=18), and a tetraploid subspecies (n=36), ssp. *circumvagum* Mosquin. Within ssp. *angustifolium*, Scoggan (1978) recognized three forms: form *angustifolium* with roseate to purplish petals; form *albiflorum* (Dum.) Haussk. with both

white sepals and petals; and form *spectabile* (Simmons) Fern. with red sepals and white petals.

E. angustifolium occurs mostly as a pioneer and sometimes as a dominant weed in disturbed areas (Moss, 1936; Broderick, 1990; Wood and Morris, 1990) which include clear-cut areas (Moss, 1936; Dyrness, 1973; Léveillée and Laberge, 1984), forest fire sites (Moss, 1936; Marie-Victorin; 1964; Wood and Morris, 1990), roadsides, and some urban areas (Gardner, 1973). Although its occurrence appears to be restricted to the early stages of succession (Dyrness, 1973; Myerscough, 1980; Wood and Morris, 1990), the presence of persistent stands of *E. angustifolium* were reported in arctic and alpine plant communities (Wein and Bliss, 1973; Myerscough, 1980) and in some sand dune habitats in Holland (van Andel, 1975). Broderick (1990) reported that the first peak of emergence occurs in early spring (late April) in the southern regions of Canada. The peaks of vegetative growth, flowering, and seed dissemination occur in late June, July, and August, respectively (Henderson *et al.*, 1979; Broderick, 1980). For the more northerly parts of Canada, phenological progression begins one month later (Broderick, 1990). Some observations suggest that the phenological development of *E. angustifolium* might begin earlier in more disturbed habitats (Henderson *et al.*, 1979; Broderick, 1980).

Although *E. angustifolium* tolerates a broad spectrum of climatic conditions (Myerscough, 1980), laboratory experiments have shown that dense shade can affect the rate of development. However, its growth form appears to be determined by an autonomous rhythm independent of daylength (Myerscough and Whitehead, 1967). Usually, high moisture levels limit its growth (Etherington, 1984) and therefore, *E.*

angustifolium is most commonly found in freely drained sites, often sandy loams (Myerscough, 1980), although its establishment may occur in sites of high surface moisture (Wood and Morris, 1990). This species appears to grow best on fertile soils and tolerates a wide pH range (Myerscough and Whitehead, 1967; van Andel *et al.*, 1978). According to Myerscough and Whitehead (1966), factors operating through the water relations of the species along the southern limits and those operating through the temperature relations and length of season growth in the northern limits appear to limit its geographical distribution.

E. angustifolium seeds are not innately dormant. Up to 100% of the seeds from newly opened capsules germinated within 10 days of exposure to warm and moist conditions under laboratory conditions (Jobidon, 1986). In controlled environments, Myerscough and Whitehead (1966) found that the optimum temperature for germination was between 20 and 30 C. Light was found to play an important role in the germination of *E. angustifolium* seeds but this requirement was removed at temperatures close to 30 C. Successful establishment from seed is generally confined to open moist sites of at least moderate fertility, on or close to the soil surface (Myerscough, 1980; Oberbauer and Miller, 1982). Although few studies have been conducted to evaluate the viability of *E. angustifolium* seeds, they appear to be characterized by short longevity. Usually, *E. angustifolium* seeds lose viability after about 18 months when stored dry at room temperature and after two years under dry storage at 5 C (Myerscough, 1980). Laboratory germination trials using other ecotypes have shown that seed viability can range from 33%

to 95% depending on the year and time of collection, and incubation conditions (Wood and Morris, 1990).

As reported by Broderick (1990), *E. angustifolium* is self-fertile although the protandrous flowers appear to promote cross-pollination, and insects appear to be the principal pollinators as the grains are usually attached to the anther by vicine threads. The plant produces numerous (about 80 000) light, plumed seeds, that are well adapted for wind dispersal (Solbreck and Andersson, 1987). However, within mature stands, population maintenance and expansion is dependant largely on vegetative growth (Broderick, 1990). Vegetative propagation occurs through the development of adventitious buds on roots when plants have reached the rosette stage. Consequently, the rosette may be considered as the establishment stage of the plants (van Andel, 1975).

As stated above, *E. angustifolium* occurs in many communities and is an important component in two major types of vegetation, the first transient and the second persistent (Myerscough, 1980). In Canada, it is frequently associated with *Betula alleghaniensis* Britton, *B. papyrifera* Marsh., *Rubus idaeus* L., *Acer rubrum* L., and some herbaceous species including *Anaphalis margaritaceae* (L.) Benth. & Hook., *Carex* spp., and *Salix* spp. (Léveillé and Laberge, 1984). Plant and tree species of economic importance also found in association with *E. angustifolium* are: *Vaccinium* spp., *Picea* spp., *Pinus* spp., *Abies balsamea* L., *Acer saccharum* Marsh., and *Pseudotsuga menziesii* (Mirbel) Franco (Broderick, 1990; Bell, 1991).

Although several vegetation control measures are currently available to forest managers, the control of *E. angustifolium* mainly relies on chemical herbicides. Prescribed

burning, for example, may lead to an inefficient control of *E. angustifolium* in part due to its high moisture content (Haeussler and Coates, 1986). In one study, *E. angustifolium* constituted only 4% of the total pre-burn understory production of an aspen stand, but it increased dramatically by the first post-burn year and by the third post-burn year, it averaged 45% of the understory (Bartos and Mueggler, 1981). Similarly, mechanical site preparation has shown to be an inappropriate control measure. By reducing competition from other species and providing a suitable seedbed for *E. angustifolium*, such practices often lead to a favourable environment for the establishment of *E. angustifolium* (Watson *et al.*, 1980; Hamilton and Yearsley, 1988).

In 1988, a biological control program against *E. angustifolium* was initiated in the Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, Québec. Thirteen pathogenic fungi were isolated from diseased plants of *E. angustifolium* and one, *Colletotrichum dematium* (Pers. ex Fr.) Grove, causing necrotic lesions on leaves and stems of infected plants, was selected for further study (Winder and Watson, 1994). The work by Winder and Watson (1994) on inoculum production, formulation, host range testing and weed control efficacy demonstrated the potential of this fungal pathogen as a biocontrol agent for *E. angustifolium*.

The objectives of this research were to: (1) determine the taxonomic status of the potential biocontrol agent; (2) determine accurately both limiting and optimum conditions for disease development; (3) maximize disease expression through a suitable formulation; (4) determine the host specificity of the pathogen; and (5) evaluate its potential to control *E. angustifolium* under field conditions.

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Chapter 2

Taxonomic status of *Colletotrichum dematium*, a bioherbicide candidate for *Epilobium angustifolium*

2.1. Abstract

The morphology of conidia and appressoria, cultural characteristics, and virulence of a bioherbicide candidate of *Epilobium angustifolium*, *Colletotrichum dematium*, were compared with other *C. dematium* isolates in an attempt to confirm its taxonomic position and possible placement as a *forma specialis* of *E. angustifolium*. Colony morphology was extremely variable among the isolates studied, and the isolate from *E. angustifolium* was characterized by the presence of dark mouse gray concentric zones. This was the only isolate of *C. dematium* evaluated that produced sclerotia with setae in culture. Within the temperature range tested, fastest growth occurred when PDA cultures of the isolate from *E. angustifolium* were incubated at 27 C. Fastest growth was achieved at this temperature for only three other isolates, whereas a temperature of 24 C was optimal for the rest of the isolates studied. Conidia of the *E. angustifolium* isolate averaged 26.9 x 3.5 µm, and were significantly longer ($P < 0.05$) than those of all other isolates studied. Only the conidia of three isolates, including the isolate from *E. angustifolium*, exhibited acute ends whereas the remaining isolates of *C. dematium* were characterized by rounded ends. Appressoria of the isolate from *E. angustifolium* were usually long-clavate, with crenate-multilobed edges, averaging 17.5 x 8.9 µm, which departed from appressoria of all other isolates

studied and general descriptions found in the literature. The appressorial characteristic of the *E. angustifolium* isolate more closely resembled the description of *C. truncatum*. Of the five *C. dematium* tested, only two, including the isolate from *E. angustifolium*, were pathogenic to *E. angustifolium*. Although sharing some of the characteristics of the species *dematium*, the isolate from *E. angustifolium* presents quite distinct features that strongly suggest that this isolate would be indeed a *forma specialis*. Therefore, it is proposed that the isolate of *C. dematium* from *E. angustifolium* be described as *Colletotrichum dematium* f .sp. *epilobii*.

2.2. Introduction

In 1988, an anthracnose-inducing pathogen was isolated from *Epilobium angustifolium* L. (Onagraceae) (fireweed), a major weed species within Canadian reforestation areas, and subsequently studied as a potential bioherbicide for the control of this weed. Based on both conidial and appressorial characteristics, the causal organism was tentatively identified as *Colletotrichum dematium* (Pers. ex Fr.) Grove (ATCC 20981) (Winder and Watson, 1994).

Under controlled environment conditions, this fungal isolate causes severe foliar lesions on *E. angustifolium* seedlings. However, in previous host range testing, pathogenicity of this *C. dematium* isolate from *E. angustifolium* was restricted to some *Epilobium* species, although this fungal species is reported as the causal agent of diseases found on a wide range of distantly related plants (Sutton, 1992). Such findings, along with its morphological characteristics and the absence of records of form-species with similar

characteristics within the Onagraceae family, suggested the possibility that this isolate could be a *forma specialis* (Winder and Watson, 1994).

This study was conducted with the objective to examine more fully this fungal isolate from *E. angustifolium* using a combination of the most common descriptive criteria for *Colletotrichum* taxonomy, which included conidial, appressorial, and cultural characteristics, as well as temperature response (Sutton, 1980; Smith, 1990; Walker *et al.*, 1991; Gunnell and Gubler, 1992; Denoyes and Baudry, 1995). Cultural characteristics, morphology, and virulence of the *E. angustifolium* isolate was compared with other *C. dematium* isolates.

2.3. Materials and methods

2.3.1. *Fungal cultures*.

The isolate of *Colletotrichum dematium* (ATCC 20981) from *E. angustifolium* was originally isolated from diseased plants collected in the Rimouski area of Québec in 1988 and stock cultures were maintained at 4 C on potato dextrose agar (PDA; Difco, Detroit, MI) slants (Winder and Watson, 1994). Seven other isolates of *C. dematium* were obtained from different sources and maintained on PDA slants at 4 C (Table 1). Starter cultures were prepared by aseptically transferring small mycelium pieces from stock cultures to PDA plates and storing at 24 C in the dark.

2.3.2. *Cultural characteristics and temperature response*.

Cultural characteristics of each isolate were determined on PDA. A mycelial agar plug (6-mm-diameter) was removed from the leading edges of 7-day-old starter colonies

Table 1. Origin of *Colletotrichum dematium* isolates studied.

Isolate	Host plant
ATCC[x] 20981	*Epilobium angustifolium*
ATCC 24488	*Beta vulgaris*
ATCC 38107	*Lycopersicon esculentum*
ATCC 44202	*Allium cepa*
OCTC[y] 147549	n.a.[z]
OCTC 188752	n.a.
OCTC 188792	n.a.
OCTC 190485	n.a.

[x] ATCC = American Type Culture Collection.

[y] OCTC = Ottawa Culture Type Collection.

[z] Not available.

and centrally placed in plastic petri dishes containing approximately 15 ml of PDA. Colonies were incubated in triplicate for seven days at 24 C under 12 h fluorescent light. Colour designations were made according to Rayner (1970). The experiment was performed twice.

The effect of temperature on growth of each isolate was evaluated. Colonies were produced according to the methodology described above and placed in the dark at 18, 21, 24, or 27 C. Colony diameters of three replicate plates of each isolate were recorded after five days of growth at each temperature, and the experiment was carried out twice.

2.3.3. *Conidial characteristics*.

Colonies were produced according to the methodology described above and each isolate was grown on PDA for 14 days under 12 h fluorescent light at 24 C in an incubator. Conidia were harvested after 14 days, mounted in 0.2% acid-fuchsin in lactophenol, and observed at 40X with the aid of a light microscope. Conidial size of each isolate was determined by measuring the length and width of 25 randomly chosen conidia from each plate of each isolate using the Leco 2001 Image analysis system (Leco Corporation, St-Joseph, MI). All isolates were cultured in triplicate, and the study was conducted twice.

2.3.4. *Appressorial characteristics*.

Appressoria were studied using a slide culture method modified from Sutton (1962). A mycelial plug (6-mm-diameter) was removed from the margin of a 7-day-old starter colony, placed on a sterilized slide, and covered with a sterile coverslip. The slide

culture was held in a sealed glass petri dish for seven days at 24 C under 12 h fluorescent light. Appressoria were mounted in 0.2% acid-fuchsin in lactophenol and examined at 40X with the aid of a light microscope. Appressoria size of each isolate was determined by measuring the length and width of 25 randomly chosen appressoria from each slide of each isolate using the Leco 2001 Image analysis system. Each isolate included two replicates, and the study was conducted twice.

2.3.5. *Virulence of isolates to Epilobium angustifolium.*

The virulence of some of the *C. dematium* isolates on *E. angustifolium* was evaluated under controlled environment conditions. Plants were grown from seeds collected in 1993 from a population in the Beauce region of Québec. Seeds were placed in a covered tray filled with moist potting medium (Promix, Premiers Brands, New Rochelle, NY) and the trays were placed in a growth cabinet (24/16 C day/night, 300 $\mu Em^{-2}s^{-1}$, 12 h photoperiod). Two weeks after emergence, seedlings were transplanted individually into 10-cm-diameter plastic pots under the same growth conditions and watered with tap water every two to three days.

Tested isolates of *C. dematium* included ATCC 38107, ATCC 44202, OCTC 188752, OCTC 188792, and the isolate from *E. angustifolium* (ATCC 20981). The remaining isolates of *C. dematium* were not included in the present experiment due to their inability to produce sufficient amount of conidia. Conidia were produced on PDA plates according to the method previously described. After 14 days of incubation, conidia were collected from plates by adding distilled water, rubbing the culture surface gently with a plastic spatula, and filtering through four layers of cheesecloth. Conidia collected in the

filtrate were pelleted by centrifugation at 2500 x *g* for 10 min. Inoculum concentration was determined with the aid of a haemocytometer, and adjusted to a density of $1x10^8$ spores m^{-2} with distilled water. Tween 80 (polyoxyethylene sorbitan mono-oleate) (0.05% v/v) was added to the inoculum suspensions as a wetting agent.

Inoculations were performed on 4-wk-old *E. angustifolium* seedlings (4-6 true leaves) at conidia density of $1x10^8$ conidia m^{-2} using a spray chamber (Research Instrument Manufacturing Co., Guelph, ON) equipped with a full cone nozzle (TG 0.7), at 200 kPa air pressure, a speed of 1 km h^{-1}, and a spray volume of 330 L ha^{-1}. Plants were subsequently placed in a dark dew chamber (100% RH) at 24 C for 24 h and then transferred to a growth chamber having the original growth conditions.

E. angustifolium plants were monitored for disease development for a period of 14 days. Plants were rated immune (I) if no visible symptoms were observed, hypersensitive (HS) if flecking occurred, resistant (R) if only a few small necrotic lesions occurred, and susceptible (S) if extended necrotic lesions were visible within the 2-wk period. As soon as plants exhibited lesions, pieces of diseased tissues were excised, surface sterilized in 1.5% NaClO for 15 sec, rinsed in sterile distilled water, and placed on PDA to confirm the causal agent. All isolates were tested in triplicate, and the study was conducted twice.

2.3.6. *Data analysis.*

All analysis of variance tests (GLM procedure) were performed using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Treatment means were compared using the Student-Newman-Keuls Multiple Range test (SNK) at $P = 0.05$. Experiments were performed using a completely randomized design, and variance between

the two trials of each experiment were compared before pooling results by using the Chi-square test for homogeneity of variance (Gomez and Gomez, 1984). If variances were not homogeneous, results from only one experiment were presented when a similar trend was observed in both.

2.4. Results

2.4.1. *Cultural characteristics and temperature response.*

Cultural characteristics of the *C. dematium* isolates were very variable on PDA. Colour ranged from saffron (ATCC 38107) to white (OCTC 147549 and OCTC 188792) (Table 2). The cultural characteristics of the isolate of *C. dematium* from *E. angustifolium* (ATCC 20981) largely departed from those of other isolates of *C. dematium*. On PDA, colony appearance of ATCC 20981 was constantly characterized by dark mouse gray concentric zones, surrounded by a narrow margin of white to pale cream hyphae. Unlike the colonies of other *C. dematium* isolates, this isolate from *E. angustifolium* developed dark sclerotia bearing setae on PDA.

All of the isolates were included in the study of the temperature effect on radial growth (Table 3). Radial growth varied considerably among isolates at each temperature, with the least difference at 18 C and greater with increasing incubation temperature. The greatest radial growth occurred at 27 C for ATCC 38107, OCTC 147549, OCTC 188752, and ATCC 20981 from *E. angustifolium*, but 24 C was optimal for the rest of the isolates studied.

Table 2. Cultural characteristics of various *Colletotrichum dematium* isolates grown on potato dextrose agar.

Isolate	Colony appearance
ATCC 20981	dark mouse gray concentric zones, sclerotia bearing setae.
ATCC 24488	smoke gray central zone surrounded by whitish circular zones.
ATCC 38107	saffron, diffuse concentric darken zones, conidia saffron in masses.
ATCC 44202	gray olivaceous, concentric olivaceous gray zones bearing orange conidial masses.
OCTC 147549	white compact mycelium, streaked in the central zone.
OCTC 188752	central zone of white mycelium covering diffuse iron gray and smoke gray concentric zones.
OCTC 188792	white compact mycelium.
OCTC 190485	irregular greenish black central zone surrounded by rosy buff zone.

Table 3. Average colony diameter (mm) of various *Colletotrichum dematium* isolates after five days of culture on potato dextrose agar at different incubation temperatures in the dark.

Isolate	Temperature (C)			
	18	21	24	27
ATCC 20981	[y]33.4 ± 0.2[z]	43.5 ± 0.5	47.0 ± 0.4	51.6 ± 0.2
ATCC 24488	31.3 ± 0.1	33.3 ± 0.4	42.1 ± 0.6	30.6 ± 0.6
ATCC 38107	35.2 ± 0.5	41.5 ± 0.4	48.4 ± 0.2	56.0 ± 0.3
ATCC 44202	22.6 ± 0.4	24.0 ± 0.3	25.5 ± 0.6	24.6 ± 0.2
OCTC 147549	37.5 ± 0.5	47.4 ± 1.1	52.9 ± 0.4	53.1 ± 0.3
OCTC 188752	30.8 ± 0.2	38.8 ± 0.2	41.8 ± 0.2	43.5 ± 0.7
OCTC 188792	32.6 ± 0.5	39.1 ± 0.4	42.8 ± 0.4	36.2 ± 0.3
OCTC 190485	32.8 ± 0.3	41.4 ± 0.5	46.7 ± 0.3	45.8 ± 0.4

[y] Measurements represent the mean of six plate replicates.

[z] ± Standard error.

2.4.2. *Conidial characteristics*.

ATCC 38107, ATCC 44202, OCTC 188752, OCTC 188792, and ATCC 20981, the isolate from *E. angustifolium*, produced profuse conidia on PDA (Table 4). Conidial production for all other evaluated isolates was weak to none under the same growth conditions. Although sharing the typical falcate-shape of the species, conidia of the isolate of *C. dematium* from *E. angustifolium* presented distinct features. For instance, conidia were generally slightly curved and tapering to acute ends. In comparison, conidia of ATCC 38107 were strongly falcate, whereas similar structures of OCTC 188752, OCTC 188792, and OCTC 190485 exhibited rather rounded ends. Conidia of ATCC 20981 from *E. angustifolium* were significantly longer, averaging 26.9 µm. ATCC 44202 and ATCC 20981 from *E. angustifolium* were the narrowest, with an average width of 3.5 µm. Both conidia size and shape of this isolate fit the descriptions of Winder and Watson (1994), although conidia were wider in the present study.

2.4.3. *Appressorial characteristics*.

All of the isolates studied, except OCTC 190485, produced mycelial appressoria using the slide culture technique. Both appressorial shape and size of ATCC 20981 from *E. angustifolium* largely departed from the corresponding structures of the other isolates evaluated (Table 5). This isolate usually produced long clavate, sometimes short or irregular, brown mycelial appressoria with mainly crenate edges. By contrast, ATCC 24488, ATCC 38107, ATCC 44202, OCTC 147549, and OCTC 188792 produced simple to slightly lobed, mainly short clavate appressoria. Appressoria of OCTC 188752 were strongly lobed and very variable in shape. Appressorial characteristics of the studied

Table 4. Conidial characteristics and production of various *Colletotrichum dematium* isolates cultured on potato dextrose agar.

Isolate	Conidial measurements (μm)[wx]		Production[y]	Shape[z]
	Length	Width		
ATCC 20981	26.9 a	3.5 c	++	fl / ac
ATCC 24488			-	
ATCC 38107	23.1 c	3.7 b	++	fl / ac
ATCC 44202	23.3 c	3.3 d	++	fl / ac
OCTC 147549			-	
OCTC 188752	24.1 b	4.4 a	++	fl / rd
OCTC 188792	23.0 c	4.3 a	++	fl / rd
OCTC 190485	24.1 b	3.8 b	+	fl / rd

[w] Measurements represent the mean of 150 conidia per isolate.

[x] Means having the same letter in a column are not significantly different according to the Student-Newman- Keuls multiple range test (SNK) at $P = 0.05$.

[y] - = no conidia produced; + = few conidia produced; + + = many conidia produced.

[z] fl = falcate; ac = acute ends; rd = rounded ends.

Table 5. Appressorial characteristics of various *Colletotrichum dematium* isolates.

Isolate	Appressorial measurements (μm)[yz]		Shape
	Length	Width	
ATCC 20981	17.5 a	8.9 a	simple to multilobed, mainly long clavate
ATCC 24488	9.3 de	6.1 d	simple to slightly lobed, mainly short clavate
ATCC 38107	9.9 d	7.0 c	simple to slightly lobed, mainly short clavate
ATCC 44202	9.0 e	5.7 e	simple to slightly lobed, mainly short clavate
OCTC 147549	13.8 b	8.6 a	simple to slightly lobed, mainly short clavate
OCTC 188752	12.6 c	7.8 b	strongly lobed, variable in shape
OCTC 188792	13.3 bc	7.3 c	simple to slightly lobed, mainly short clavate

[y] Measurements represent the mean of 100 appressoria per isolate.

[z] Means having the same letter in a column are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.

isolates fit the description of the species by Sutton (1962, 1980), whereas appressorial size of ATCC 20981 from *E. angustifolium* averaged 17.5 x 8.9 µm. These results are quite divergent from those reported by Winder and Watson (1994) but their appressoria measurements were made directly on the leaf surface which could account for the difference. In this study, appressoria measurements for all isolates were taken from slide cultures and both the length and width of ATCC 20981 from *E. angustifolium* were significantly ($P < 0.05$) greater than corresponding measurements of the other isolates studied.

2.4.4. *Virulence of isolates to Epilobium angustifolium*.

Only plants inoculated with ATCC 20981 from *E. angustifolium* and ATCC 38107 exhibited disease symptoms and were rated as susceptible (S) (data not shown). All other isolates tested failed to induce disease symptoms and plants were rated as immune (I). Disease damage on plants inoculated with ATCC 38107 was as severe as that observed on *E. angustifolium* plants inoculated with ATCC 20981. Typical symptoms consisted of large and irregular, but not expanding, tan foliar lesions. Disease symptoms were visible as early as 36 h following inoculation, and both isolates were successfully re-isolated from the diseased plant specimens of *E. angustifolium*.

2.5. Discussion

On the basis of both conidial shape and size as well as cultural characteristics, the fungal isolate from *E. angustifolium* would justify its identification as *C. dematium* as proposed by Winder and Watson (1994), although conidia were longer than any other

isolate studied or other reported measurements for that species (Barksdale, 1972; Beraha and Wright, 1973; Maas, 1978; Sutton, 1980). Although the temperature response study did not provide a useful means of discriminating for the isolate from *E. angustifolium*, there were other distinct characteristics observed for this isolate. The fact that ATCC 20981 was the only isolate of *C. dematium* to produce sclerotia bearing setae was a distinct feature. In addition, appressorial characteristics of ATCC 20981 largely departed from the other *C. dematium* isolates studied and the reported characteristics for that species. Whereas Sutton (1980) described *C. dematium* appressoria as usually entire, ranging 8-11.5 x 6.5-8 µm in size, the appressoria of ATCC 20981 from *E. angustifolium* were characterized by usually irregular edge (crenate - multilobed) and an average size of 17.5 x 8.9 µm. Such appressorial characteristics are closer to the description for *Colletotrichum truncatum* (Sutton,1980).

Five *C. dematium* isolates, including the original isolate from *E. angustifolium* (ATCC 20981), were compared for their pathogenicity to *E. angustifolium* in an attempt to confirm, as proposed by Winder and Watson (1994), the isolate from *E. angustifolium* as a *forma specialis* of this weed species. The inoculation experiment demonstrated that, under controlled environment conditions, *E. angustifolium* was immune to most of the isolates evaluated, but ATCC 38107, a causal agent of anthracnose on tomato (*Lycopersicon esculentum*) (Stevenson *et al.*, 1978), induced indistinguishable symptoms from those caused by ATCC 20981 from *E. angustifolium*. Such variability in virulence among fungal isolates of the same species has been regularly reported (Roy, 1982; McLean and Roy, 1988; Smith, 1990; Denoyes and Baudry, 1995). As hypothesized by McLean

and Roy (1988), there is a possibility that the non-pathogenic isolates could be simply less aggressive on *E. angustifolium* and would have consequently expressed their virulence if a longer dew period incubation would have been used. Nevertheless, previous host range studies on the isolate of *C. dematium* from *E. angustifolium* have suggested that ATCC 20981 was specific to the Onagraceae family (Winder and Watson, 1994; Léger *et al.*, 1995).

The present study generally supports the identification of ATCC 20981 from *E. angustifolium* as *Colletotrichum dematium* as proposed by Winder and Watson (1994), although, some characteristics, especially appressorial shape and size, suggest that this isolate might be closer to *C. truncatum*. Although presenting the usual falcate-shape of the *C. dematium* taxa, the distinct morphological, cultural, and pathogenicity characteristics of the isolate from *E. angustifolium* (ATCC 20981) suggest that this isolate is a *forma specialis* and should be designated as *Colletotrichum dematium* f.sp. *epilobii*.

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Connecting text

Within the development phase of a bioherbicide candidate, specific attention must be paid to factors, either biotic or abiotic, that could influence the expression of the disease. In this respect, earlier studies with a formulated inoculum suspension examined some of the environmental requirements of *C. dematium* f.sp. *epilobii* to achieve a satisfactory level of control of *E. angustifolium* plants (Winder and Watson, 1994)[1]. To augment the work of Winder and Watson (1994), the present study was conducted to more fully investigate the influence of both the duration and temperature of the dew period, the amount of inoculum, and the age of the plants on the disease expression of the unformulated fungus. Extensive efforts were also placed on the investigation of suitable formulations, particularly on those that reduced dew requirement, in attempts to overcome environmental constraints on disease expression.

[1]**Winder, R.S. and Watson, A.K. 1994**. A potential microbial control for fireweed (*Epilobium angustifolium*). Phytoprotection 75:19-33.

Chapter 3.

Evaluation of the bioherbicidal efficacy of *Colletotrichum dematium* f.sp. *epilobii* for the control of *Epilobium angustifolium*

3.1. Abstract

The potential for the fungal pathogen, *Colletotrichum dematium* f.sp. *epilobii*, to control *Epilobium angustifolium*, a major pioneer weed species in coniferous forests of Canada, was investigated. Under controlled environment conditions, the effectiveness of *C. dematium* f.sp. *epilobii* was greatly affected by plant age, inoculum density, and by temperature and length of the dew period. The most severe damage was sustained by seedlings subjected to spore applications at a density of $1x10^9$ conidia m^{-2}. Susceptibility decreased as plant age increased, and older plants were unaffected by the fungus. Satisfactory levels of control were limited to long dew duration (>18 h) and high temperature treatments. Higher levels of control were constantly achieved when canola oil was added (25% v/v) to the spore inoculum. This formulation resulted in extensive necrosis, and substantial above-ground dry biomass reductions despite spraying at lower conidial densities (about 10 times less) and subjecting seedlings to lower dew durations. Buffering the fungal inoculum suspension to acidic levels (pH 3.0) increased the level of control compared with an unbuffered spore suspension, whereas the adjustment to more

alkaline pH levels using the citrate-phosphate buffer (pH > 4.0) inhibited disease expression.

3.2. Introduction

Projected increases in the worldwide demand for wood products has challenged forest managers to intensify reforestation success (Wagner and Zasada, 1991) and concomitantly, increased attention on more sustainable and efficient vegetation management practices. Non-commercial competing vegetation represents a major constraint in forest nurseries, plantations, and conifer reforestation because of the ability of these plants to compete for nutrients, water, light, and space (Watson and Wall, 1995). *Epilobium angustifolium* L. (fireweed) is an aggressive herbaceous perennial plant species that is distributed throughout the major Canadian forest areas. This forest weed species is able to rapidly colonize newly disturbed sites, forming dense stands, and reducing the growth of newly planted conifer seedlings (Dyrness, 1973; Eis, 1981).

Due to their low cost and ease of application, chemical herbicides have been traditionally used in the Canadian forest sector to control forest weeds, including *E. angustifolium* (Gouvernement du Québec, 1991; Wagner and Zasada, 1991). However, several factors have necessitated the development of alternative or complementary control strategies. For example, *E. angustifolium* can rapidly dominate an area, especially during hardening-off periods when young conifer seedlings are most sensitive to herbicides. Moreover, in conjunction with demands to enhance forest management, there is increasing pressure in North America to reduce herbicide use (Walstad, 1988), and particularly in

Québec where a total ban within the forestry sector is projected by the year 2001 (Gouvernement du Québec, 1991).

The bioherbicide strategy involves the application of plant pathogenic fungi in a manner analogous to the application of chemical herbicides. In 1988, a fungus causing large necrotic lesions on *E. angustifolium* leaves and stems was isolated and tentatively identified as *Colletotrichum dematium* f.sp. *epilobii*. Preliminary studies have demonstrated the potential of this fungal pathogen as a biocontrol agent for *E. angustifolium* (Winder and Watson, 1994). Based on these findings, the objective of this study was to more thoroughly investigate the influence of inoculum density, plant age, dew duration, dew temperature, and the use of different formulations on the control of *E. angustifolium* by *C. dematium* f.sp. *epilobii*.

3.3. Materials and methods

3.3.1. *Inoculum production*.

Colletotrichum dematium f.sp. *epilobii* was originally isolated from diseased *E. angustifolium* plants collected in the Rimouski area of Québec in 1988 and stock cultures were maintained at 4 C on potato dextrose agar (PDA; Difco, Detroit, MI) slants (Winder and Watson, 1994). Starter cultures were prepared by aseptically transferring small pieces of mycelium from stock cultures to PDA plates and incubating at room temperature (21 ± 1 C) for two weeks.

Conidia of *C. dematium* f.sp. *epilobii* were produced on millet seeds (*Pennisetum glaucum* (L.) R. Br). Twenty grams of millet seeds were placed in 250 ml Erlenmeyer

flasks, moistened with 20 ml of distilled water, and autoclaved for 17 min at 100 kPa and 120 C. Two mycelial agar plugs (6-mm-diameter) were removed from the leading edges of 10- to 14-day-old starter colonies of the fungus and used to inoculate each flask of millet seed. The flasks were incubated for 14 days at room temperature (21 ± 1 C) under 12 h near ultra-violet light (NUV; J-05, UVP, Inc., Circleville, OH), and shaken every two to three days to prevent grain aggregation. Conidia were collected with the addition of 50 ml distilled water to the flasks, shaking the flasks using a rotary shaker at 250 rpm for 30 min, and filtering through a nylon mesh. Conidia collected in the filtrate were pelleted by centrifugation at 2500 x *g* for 10 min. Inoculum concentration was determined with the use of a haemocytometer, and adjusted to the desired density with distilled water. Tween 80 (polyoxyethylene sorbitan mono-oleate) (0.05% v/v) was added to the inoculum suspensions as a wetting agent.

3.3.2. *Plant production*.

E. angustifolium seed was collected in 1993 from a population in the Beauce region of Québec and the pappus removed by sieving through a 425 µm soil sieve. Seeds were placed in a covered tray filled with moist potting medium (Promix, Premiers Brands, New Rochelle, NY) and placed in a growth cabinet (24/16 C day/night, 300 $\mu Em^{-2}s^{-1}$, 12 h photoperiod). Two weeks after emergence, seedlings were transplanted individually into 10-cm-diameter plastic pots under the same growth conditions and watered with tap water every two to three days.

3.3.3. *Inoculation procedure and weed control assessment.*

Unless otherwise stated, all inoculations were performed on 4-wk-old *E. angustifolium* seedlings (4-6 true leaves) at a conidia density of $1x10^9$ conidia m^{-2} using a spray chamber (Research Instrument Manufacturing Co., Guelph, ON) equipped with a full cone nozzle (TG 0.7), at 200 kPa air pressure, a speed of 1 km h^{-1}, and a spray volume of 330 L ha^{-1}. Plants were subsequently placed in a dark dew chamber (100% RH) at 24 C for 24 h and then transferred to a growth chamber having the original growth conditions.

One week following inoculation, *E. angustifolium* seedlings were harvested. Live plants were cut at the soil line and above-ground biomass was determined by drying excised living aerial tissue for three days at 60 C, and weighing.

3.3.4. *Plant age and inoculum density.*

E. angustifolium plants at each of four growth stages (4, 7, 10, or 13-wk-old) were sprayed at three inoculum densities ($1x10^7$, $1x10^8$, or $1x10^9$ conidia m^{-2}) using a factorial design. Control plants were sprayed only with water and the surfactant (Tween 80 at 0.05% v/v). Following dew incubation, *E. angustifolium* plants were returned to the growth chamber. Based on data from the formulation study, this experimental setup was repeated using a spore suspension formulated in an oil emulsion (25% canola oil v/v). Treatment controls for the latter experiment were sprayed with the oil emulsion only.

3.3.5. *Dew duration and dew temperature.*

After inoculation, *E. angustifolium* seedlings were placed in a dark dew chamber for 6, 12, 18, or 24 h at temperatures of 15, 20, 25, or 30 C and then returned to original

growth conditions. Five plants within each treatment combination were sprayed with water and surfactant only and served as controls. Since the temperature treatment was evaluated over time using different conidial suspensions, the percentage spore germination for each inoculation was estimated in order to determine any variation due to inoculum application. Percentage spore germination was estimated by inoculating three water agar plates with 1.0 ml of the conidial suspension and counting the number of spores that had germinated after 12 h. A similar experiment was carried out using a spore suspension formulated using an oil emulsion (25% canola oil v/v), and treatment controls were included by spraying with the oil emulsion alone.

3.3.6. *Effect of various additives on disease expression.*

E. angustifolium seedlings were inoculated with five different formulations of *C. dematium* f.sp. *epilobii* using a conidia density of $1x10^9$ conidia m^{-2}: 1% Metamucil (w/v) (Procter & Gamble Inc., Toronto, ON), 1% gelatin (w/v) (BDH Chemicals, Toronto, ON), 20% Sorbo™ (v/v) (64% sorbitol, Aktemix Inc., Brantford, ON), 1% gelatin with 20% Sorbo, and 25% canola oil emulsion (v/v). The oil formulation was prepared in a stainless steel semimicro jar blender (Thomas Scientific, Swedesboro, NJ) by mixing at high speed for 30 sec the canola oil and 0.1% soybean lecithin (w/v). The aqueous phase, containing the spore suspension, was added to the oil phase and mixed for an additional 30 sec. A set of plants inoculated with water and surfactant only served as controls. Following inoculation, plants were subjected to an 18-h dew in a dark dew chamber and then returned to original growth chamber conditions.

3.3.7. *Effect of oil emulsion concentration and dew duration*.

E. angustifolium seedlings were grown from seeds on a growth bench under the same conditions previously described. Conidial suspensions of *C. dematium* f.sp. *epilobii* were amended with four different concentrations of canola oil emulsion: 0%, 6.25%, 12.5%, or 25%. The oil formulation was prepared according to the method previously described. Following inoculation, *E. angustifolium* seedlings were placed in a dark dew chamber for 0, 6, 12, 18, or 24 h, and then returned to original growth bench conditions. The control treatment consisted of spraying plants with the oil emulsion only.

3.3.8. *Effect of inoculum suspension pH and buffers*.

Subsequent to spore harvesting, the pH of the *C. dematium* f.sp. *epilobii* conidial suspension was adjusted to five different pH values (3.0, 4.0, 5.0, 6.0, or 7.0) using two buffers: citrate-phosphate (0.1M $C_6H_8O_7 \cdot H_2O$ - 0.2M Na_2HPO_4) and MES-NaOH (0.1M 2-(N-morpholino)ethanesulfonic acid - 0.1M NaOH). The buffer solutions were prepared using standard procedures (Dhingra and Sinclair, 1985). Several drops of HCl (0.1M) were added to the conidial suspension to attain the lowest pH value (3.0) used in this experiment. Each pH treatment evaluated included a set of seedlings sprayed only with a buffer solution without conidia that served as controls. Additionally, an unbuffered spore suspension of *C. dematium* f.sp. *epilobii* was applied to seedlings at a rate of $1x10^9$ conidia m^{-2} and served as a comparative treatment.

3.3.9. *Data analysis*.

All statistical analyses were performed using the Statistical Analysis System (SAS

Institute, Inc., Cary, NC). The effect of *C. dematium* f.sp. *epilobii* on the above-ground biomass of *E. angustifolium* was expressed as the percentage of healthy shoot biomass (% HSB). The % HSB was calculated by dividing the dry weight of living aerial parts of inoculated plants by the mean dry weight of living aerial parts of their respective uninoculated controls, and multiplying by 100. Logarithmic transformation [log (% HSB + 1)] was used to obtain homogeneity of variance.

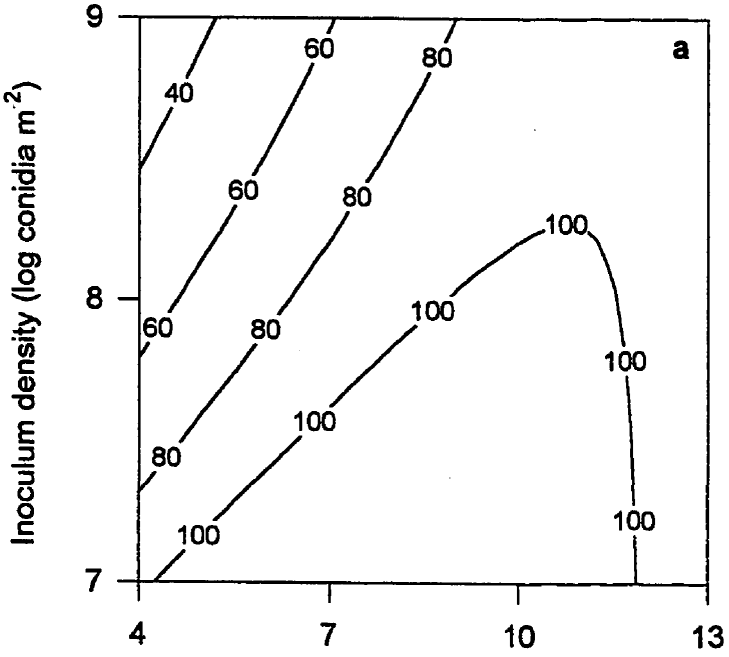
All experiments were performed twice with five replicates per treatment. The dew duration and temperature experiments were conducted in a randomized complete block design while the other experiments were conducted in completely randomized design. Both the formulation and the pH experiments were subjected to analysis of variance (GLM procedure) and treatment means were compared using the Student-Newman-Keuls Multiple Range test (SNK) at $P=0.05$. Regression equations were generated from factorial experiments using the least-squares regression method (GLM procedure). Variance between the two trials of each experiment were compared before pooling results by using the Chi-square test for homogeneity of variance (Gomez and Gomez, 1984). If variances were not homogeneous, results from only one experiment were presented when a similar trend was observed in the other.

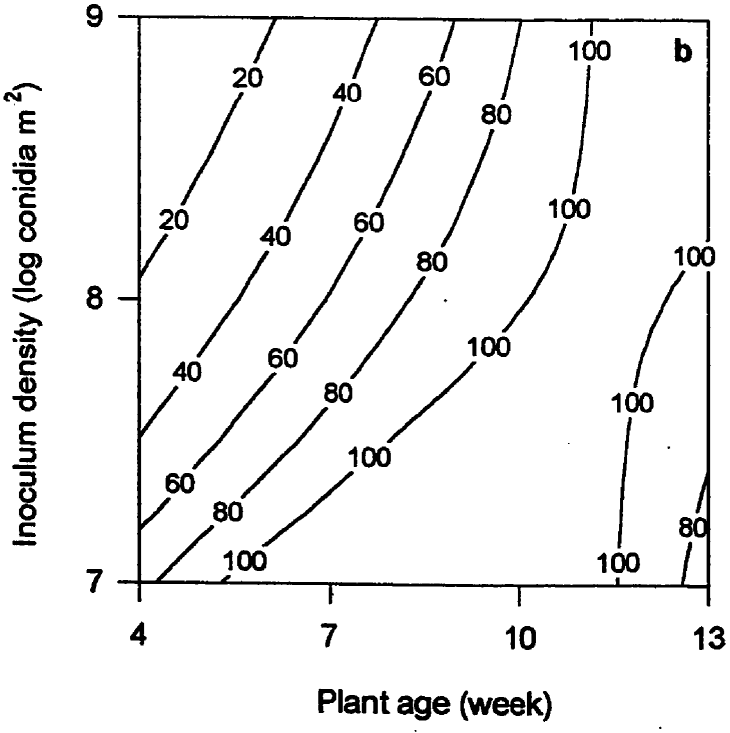
3.4. Results

3.4.1. *Plant age and inoculum density*.

The contour projection curves showed that the disease impact of an unformulated conidial suspension of *C. dematium* f.sp. *epilobii* on *E. angustifolium* biomass was significantly affected by plant age ($P < 0.001$) (Fig. 1a). Within the range of inoculum

Figure 1. Contour projection curves for the effect of inoculum density and plant age on percentage healthy-shoot biomass (% HSB) of *Epilobium angustifolium* based on shoot biomass of uninoculated control plants (i.e. 100% HSB) for plants subjected to **(a)** an unformulated *Colletotrichum dematium* f.sp. *epilobii* conidial suspension and **(b)** a conidial suspension of *C. dematium* f.sp. *epilobii* amended with 25% canola oil (v/v). Plants were provided with a 24 h dew in the dark at 24 C. Regression equations are expressed in the form log (Z+1), where Z = %.HSB (i.e. controls), A = plant age, and D = inoculum density. For the unformulated inoculum treatment: $Z = 4.3261 - 0.1025A - 0.3856D + 0.0319AD - 0.0075A^2$ ($r^2 = 0.68$) and for the formulated inoculum treatment: $Z = 6.8794 - 0.2829A - 0.8081D + 0.0712AD - 0.0128A^2$ ($r^2 = 0.70$).


a
Inoculum density (log conidia m-2)
9
8
7
4
7
10
13
40
60
80
100


b
Inoculum density (log conidia m-2)
Plant age (week)
9
8
7
4
7
10
13
20
40
60
80
100

densities used, above-ground biomass decreased quadratically with decreasing plant age and the most severe damage occurred when the fungus was applied on 4-wk-old plants.

Above-ground biomass was also significantly affected by inoculum density (P = 0.001). When fungal inoculum was applied on younger plants, above-ground biomass decreased linearly with increasing inoculum density, and the most severe disease damage occurred following an application of $1x10^9$ conidia m^{-2}. Although damage was nearly absent on 4-wk-old *E. angustifolium* plants sprayed at a density of $1x10^7$ conidia m^{-2}, the above-ground biomass for plants subjected to *C. dematium* f.sp. *epilobii* spore applications of $1x10^8$ and $1x10^9$ conidia m^{-2} was reduced by 48% and 71%, respectively. A similar pattern was also observed for 7-wk-old plants, however, higher inoculum densities were generally necessary to achieve similar reductions in biomass. For instance, the application of $1x10^9$ conidia m^{-2} on 7-wk-old plants resulted in a biomass decrease of 41% which was nearly identical to the biomass reduction sustained with an application of $1x10^8$ conidia m^{-2} on 4-wk-old plants. However, disease did not develop on older plants, even when subjected to the highest inoculum density applications. Hence, the interaction between plant age and inoculum density was found to be highly significant in this experiment ($P < 0.001$).

The contour projection curves demonstrated that the effect of *C. dematium* f.sp. *epilobii* on E. *angustifolium* above-ground biomass was generally greater following the amendment of 25% canola oil to the inoculum (Fig. 1b). However, the disease damage pattern was similar to that for the unformulated inoculum application. All of the estimated variables as well as their interaction remained highly significant ($P < 0.001$). Above-ground

biomass decreased quadratically with decreasing plant age with 4-wk-old plants being the most susceptible. However, 10- and 13-wk-old *E. angustifolium* plants subjected to the formulated conidial suspension remained generally unaffected by the fungus.

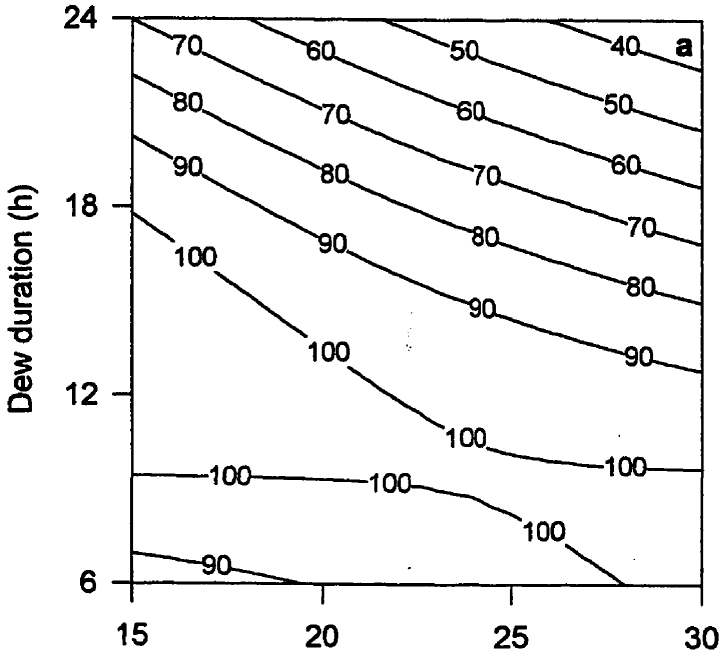
E. angustifolium above-ground biomass decreased linearly with increasing inoculum density of the oil-based formulation, and as with the unformulated inoculum application, the most severe damage was sustained when younger plants were sprayed at a density of $1x10^9$ conidia m^{-2}. For example, the application of $1x10^7$ conidia m^{-2} on 4-wk-old plants resulted in a 25% reduction in shoot biomass, whereas a 94% reduction was obtained following the application of $1x10^9$ conidia m^{-2}.

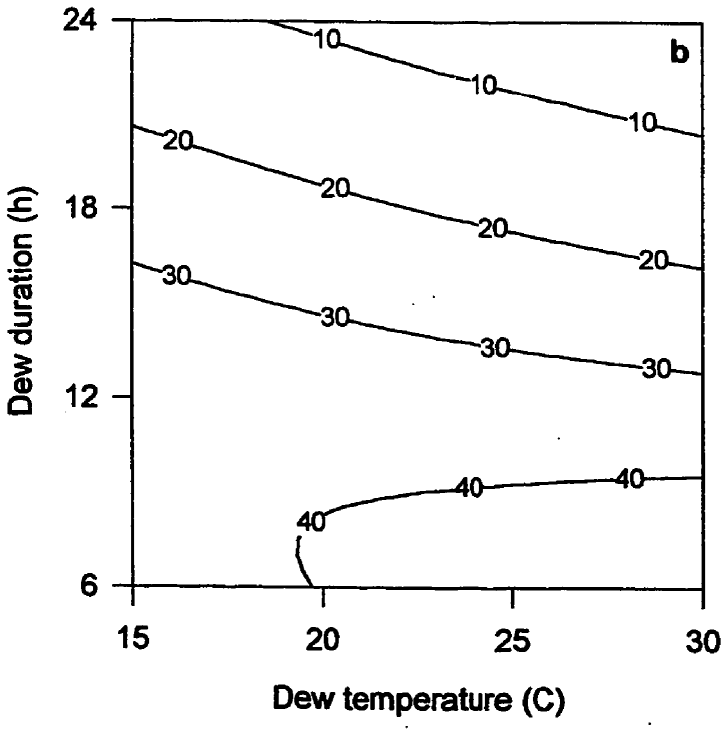
Using the oil emulsion formulation, an inoculum density 10 times lower than that used for the inoculum application alone was required to achieve comparable reductions in shoot biomass of younger plants. For example, while a 39% reduction in the biomass of 7-wk-old plants was obtained when *C. dematium* f.sp. *epilobii* was applied at a rate of $1x10^8$ conidia m^{-2} in conjunction with the oil emulsion, $1x10^9$ conidia m^{-2} were necessary to achieve similar biomass reductions when applied without the addition of oil. A similar trend was also observed for treatment applications on 4-wk-old plants.

3.4.2. *Dew duration and dew temperature.*

The contour projection curves generated from the model showed that both the length and temperature of dew period, as well as their interaction, significantly affected *C. dematium* f.sp. *epilobii* disease expression when the fungus was applied unformulated ($P < 0.05$) (Fig. 2a). Hence, within the dew temperature range tested, above-ground biomass of *E. angustifolium* seedlings decreased quadratically with an increase in dew duration. Dew

Figure 2. Contour projection curves for the effect of dew duration and temperature on percentage healthy-shoot biomass (% HSB) of *Epilobium angustifolium* based on shoot biomass of uninoculated control plants (i.e. 100% HSB) for plants subjected to (**a**) an unformulated *Colletotrichum dematium* f.sp. *epilobii* conidial suspension and (**b**) a conidial suspension of *C. dematium* f.sp. *epilobii* amended with 25% canola oil (v/v). Inoculations were performed on 4-wk-old plants using a density of $1x10^9$ conidia m^{-2}. Regression equations are expressed in the form log (Z +1), where Z = % HSB (i.e. controls), T = dew temperature, and D = dew duration. For the unformulated inoculum treatment: $Z = 1.5044 + 0.0143T + 0.0688D - 0.0015TD - 0.0017D^2$ ($r^2 = 0.37$) and for the formulated inoculum treatment: $Z = 1.1328 + 0.0192T + 0.0670D - 0.0019TD - 0.0021D^2$ ($r^2 = 0.49$).


a
Dew duration (h)
24
18
12
6
15
20
25
30
70
60
50
40
80
70
60
50
90
80
70
60
100
90
80
70
100
90
80
100
100
90
100
100
100
90


b
Dew duration (h)
Dew temperature (C)
24
18
12
6
15
20
25
30
10
10
10
20
20
20
20
30
30
30
30
40
40
40
40

durations greater than 12 h were consistently required to induce biomass losses, and the most severe levels of damage were typically observed following a 24-h dew period. In contrast, the fungus caused above-ground biomass reductions at all dew temperatures tested, with the greatest reductions (69%) occurred with *E. angustifolium* seedlings subjected to a dew temperature of 30 C for 24 h. However, for each dew temperature, disease expression varied depending on the length of the dew period. For instance, no reduction in shoot biomass was observed when inoculated *E. angustifolium* seedlings were incubated for 18 h at 15 C and the dew duration requirement to initiate biomass reduction was reduced to 12 h at 30 C. In general, above-ground biomass decreased linearly with increasing dew temperature. The absence of control for low dew duration might explain the low r^2 obtained for the model.

As expected, greater reductions in seedling above-ground biomass were consistently achieved following the application of *C. dematium* f.sp. *epilobii* in combination with 25% canola oil (Fig. 2b) and only the dew duration, with a quadratic relationship, remained significant ($P < 0.05$). In the presence of oil, the dew length requirement was considerably reduced and substantial biomass reductions were observed for all dew durations tested. For example, decreases in above-ground biomass ranging from 37 to 48% were obtained following dew periods as short as 6 h for all temperatures examined. For low dew durations (< 12 h), disease development appeared to have been stimulated by cooler temperatures. However, for seedlings subjected to longer dew durations, greater damage was sustained as temperatures increased. As was observed for

the unformulated inoculum suspension, the greatest level of control (95% biomass reduction) occurred with 24-h dew at 30 C.

3.4.3. *Effect of various additives on disease expression.*

All of the additives tested improved to various extent *C. dematium* f.sp. *epilobii* disease expression (Fig. 3). Although a slight reduction in above-ground biomass (10%) was observed when *E. angustifolium* seedlings were sprayed with an unformulated *C. dematium* f.sp. *epilobii* conidial suspension, consistently greater biomass reductions were obtained when the conidial suspension was applied in combination with Metamucil (31%), Sorbo (26%), gelatin (36%), and with a mixture of gelatin and Sorbo (37%). However, the oil-based emulsion was the most effective formulation studied, significantly reducing the above-ground biomass of *E. angustifolium* plants by 51% ($P < 0.05$).

Disease development was very rapid for seedlings subjected to the oil-based emulsion, with symptoms appearing as early as 18 h following inoculation. Typical lesions, characterized by translucent plant tissue, were restricted to leaves and resulted in large, dark necrotic areas, 36 h after inoculation. In contrast, when *C. dematium* f.sp. *epilobii* was applied alone, disease symptom development was slower and less severe. Collapsed leaf tissues were not observed, but only irregular and limited tan foliar lesions developed.

Some phytotoxic effects (e.g. reddish and yellow spots, and occasional leaf burning) were observed on oil-treated plant controls. However, these symptoms did not have any significant impact on the above-ground biomass of *E. angustifolium* seedlings. Based on these observations, the vegetable oil amendment of the *C. dematium* f.sp. *epilobii* conidial suspension was chosen for subsequent studies.

Figure 3. Effect of various *Colletotrichum dematium* f.sp. *epilobii* formulations on percentage healthy-shoot biomass (% HSB) of *Epilobium angustifolium* based on shoot biomass of uninoculated control plants (i.e. 100% HSB). Treatment 1 = conidia + water; 2 = conidia + metamucil (1%); 3 = conidia + gelatin (1%); 4 = conidia + sorbo (20%); 5 = conidia + gelatin (1%) + sorbo (20%); and 6 = conidia + canola oil (25%). Inoculations were performed on 4-wk-old plants at a density of $1x10^9$ conidia m^{-2}. Following inoculations, plants were provided an 18 h dew at 24 C. Columns represent the means of five plants. Columns having the same letters are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.

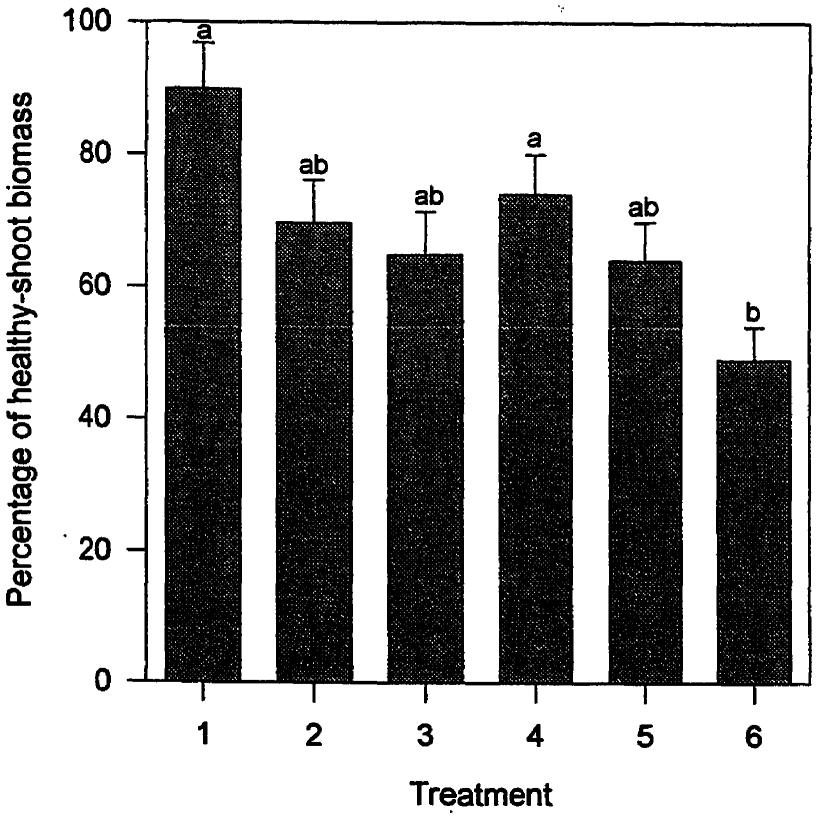
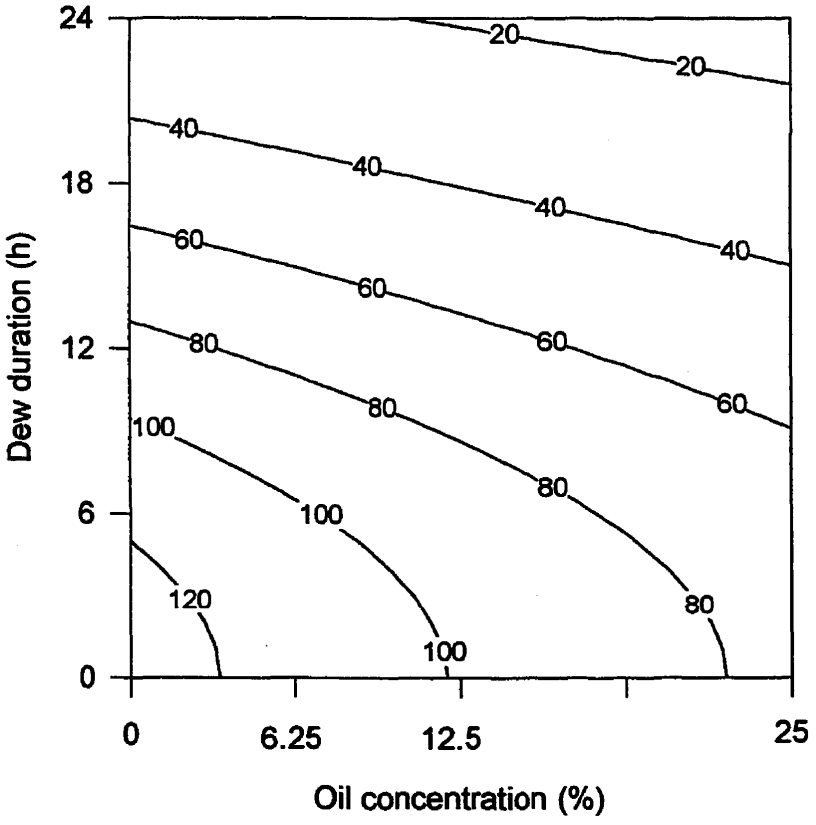

Percentage of healthy-shoot biomass
100
80
60
40
20
0
a
ab
ab
a
ab
b
1
2
3
4
5
6
Treatment

Figure 4. Contour projection curves for the effect of oil concentration and dew duration on percentage healthy-shoot biomass (% HSB) of *Epilobium angustifolium* based on shoot biomass of uninoculated control plants (i.e. 100% HSB). *Colletotrichum dematium* f.sp. *epilobii* inoculation was performed on 4-wk-old plants at a density of $1x10^9$ conidia m^{-2}, and plants were provided a dew temperature of 24 C. The regression equation of the form log (Z +1) was: 2.1138 - 0.0091C - 0.0002D - 0.0012D^2 (r^2 = 0.66), where Z = % HSB (i.e. controls), C = oil concentration, and D = dew duration.


24
18
12
6
0
Dew duration (h)
20
20
40
40
40
40
60
60
60
60
80
80
80
80
100
100
100
120
0
6.25
12.5
25
Oil concentration (%)

3.4.4. *Effect of oil emulsion concentration and dew duration.*

Both the concentration of the oil-based formulation of *C. dematium* f.sp. *epilobii* and dew duration had a significant effect on the disease expression of the fungus ($P < 0.001$). Within the range of oil concentrations tested, contour projection curves showed that the above-ground biomass of *E. angustifolium* seedlings decreased quadratically with increasing dew duration, with the highest reductions observed for dew lengths of 24 h (Fig. 4). Similarly, increasing oil concentration linearly decreased seedling above-ground biomass. The most severe damage was generally obtained when the fungus was applied in conjunction with 25% oil even in the absence of dew. Despite the lower level control achieved, similar trends were observed when this experiment was repeated.

3.4.5. *Effect of inoculum suspension pH and buffers.*

Both the pH and buffer influenced the ability of *C. dematium* f.sp. *epilobii* to infect *E. angustifolium* seedlings. For both buffers, decreasing the conidial suspension pH generally resulted in increased fungal disease expression with the highest level of control of *E. angustifolium* biomass at pH 3.0 (Table 1). The adjustment of the conidial suspension at this acidic pH level using the citrate-phosphate buffer provided a significant level of control.

At pH 3.0, disease development was consistently more severe than that sustained in the presence of an unbuffered conidial suspension. Disease developed more rapidly with symptoms being apparent as early as 24 h following inoculation. Symptoms consisted of well-defined foliar dark areas, which were absent with unbuffered conidial suspension. Treatment controls were free of any phytotoxic effects.

Table 1. Effect of various *Colletotrichum dematium* f.sp. *epilobii* inoculum pH levels using citrate-phosphate buffer and MES-NaOH buffer on *Epilobium angustifolium* healthy-shoot biomass (% HSB).[v]

pH level	Buffer		
	Citrate-phosphate	MES-NaOH (trial #1)[w]	MES-NaOH (trial #2)[w]
3.0	31 c[xyz]	20 b[xy]	66 ab[xz]
4.0	70 b	49 ab	74 ab
5.0	105 a	70 a	86 a
6.0	92 b	64 a	84 ab
7.0	99 ab	52 ab	46 b
unbuffered inoculum (pH 5.0)	49	52	85

[v] Inoculations were performed on 4-wk-old plants at a density of $1x10^9$ conidia^{-2}. Following inoculations, plants were provided an 24 h dew at 24 C.

[w] Trials were not combined because variances were not homogenous.

[x] Means having the same letters in a column are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.

[y] Significant difference between the citrate-phosphate buffer and the first trial of the MES-NaOH buffer at $P = 0.05$, according to the t-test.

[z] No significant difference between the citrate-phosphate buffer and the second trial of the MES-NaOH buffer treatments at $P = 0.05$, according to the t-test.

Within the pH range tested, the effect of the fungus was generally lower when the conidial suspension was adjusted using the citrate-phosphate buffer as compared with the MES-NaOH buffer. Levels of control sustained by inoculum suspensions buffered with the citrate-phosphate were significantly lower ($P < 0.05$) than those achieved with the MES-NaOH buffer in the first trial (Table 1). The citrate-phosphate buffer inhibited disease development with conidial suspensions having an adjusted pH over 4.0. Although *C. dematium* f.sp. *epilobii* disease expression was reduced at higher pH values when the conidial suspension was applied in conjunction with the MES-NaOH buffer, disease symptoms were always present on *E. angustifolium* plants. In addition, conidial suspensions having an adjusted pH of 7.0 using the MES buffer provided an increase in damage in comparison with lower pH levels.

3.5. Discussion

Overall, these studies demonstrated the potential for *C. dematium* f.sp. *epilobii* to induce severe damage to *E. angustifolium* plants. Disease expression was greatly influenced by several parameters. For instance, within the range of inoculum densities tested, plants of different age were not equally susceptible to the fungus, with the most severe damage occurring on younger plants. Disease injury decreased as plant age increased, with 10- and 13-wk-old plants being relatively unaffected by the fungus. These findings for *C. dematium* f.sp. *epilobii* confirm those previously reported by Winder and Watson (1994) as well as age effects for other potential bioherbicides (TeBeest *et al.*, 1978; Walker and Riley, 1982; Boyette and Walker, 1985).

Disease level increased with increasing spore application rate. For an unformulated inoculum application of *C. dematium* f.sp. *epilobii*, a density of $1x10^9$ conidia m^{-2} was necessary to achieve a satisfactory level of control. For most of the potential bioherbicide candidates, a satisfactory level of control is dependent on a high inoculum concentration, but the inoculum densities reported here are approximately 10 to 100 times greater than optimal conidial concentrations for other *Colletotrichum* spp. (Winder and Watson, 1994). The lack of control of older *E. angustifolium* plants even at relatively high conidial densities could be due to the interference of larger upper leaves, which by collapsing onto lower leaves, prevent their full inoculation (Winder and Watson, 1994). However, in this present study, upper leaves of older *E. angustifolium* plants were generally free of necrotic symptoms, suggesting some form of physiological resistance to disease development. The susceptibility of plants to a number of pathogens changes with age, as does wax composition and deposition on leaves, which constitutes a major barrier to fungal penetration and colonization (Baker, 1974; Akai and Fukutomi, 1980), and in some cases, to spore germination and germ tube formation (Conn and Tewari, 1989). Typically, on very young leaves, wax deposition is not complete, whereas for older and mature leaves, wax deposition within the cuticle is often complete and more difficult to penetrate (Martin and Juniper, 1970; McWorther *et al.*, 1990). Differential wax deposition for younger versus older plants might have influenced disease development of *C. dematium* f.sp. *epilobii*, and could partly explain the lack of control of older *E. angustifolium* plants.

The length of dew period was found to play a significant role in the severity of fungal damage on *E. angustifolium* seedlings. Although, disease developed over the range

of temperatures tested, a dew duration of at least 18 h was necessary to induce injury, with the highest level of damage consistently achieved following a 24 h dew. Similarly, greater biomass reductions were observed as temperature increased and shorter dew lengths were adequate to achieve comparable levels of control compared with those at lower temperatures.

Although trends observed in this study were similar with those reported for other bioherbicide candidates (Walker and Riley, 1982; McRae and Auld, 1988; Heiny and Templeton, 1991), the more stringent environmental requirements of *C. dematium* f.sp. *epilobii* will limit its effectiveness, particularly in regions where the weed is a problem. The requirement of an extended dew duration to achieve maximum infection constitutes a major constraint to the development of this fungus as a bioherbicide.

The use of suitable fungal formulations constitutes a way to overcome these limitations and to enhance fungal disease expression. A number of adjuvants have been shown to increase bioherbicidal efficacy (Walker, 1981; Wymore and Watson, 1986; Ormeno-Nunez *et al.*, 1988; Quimby *et al.*, 1989). Winder and Watson (1994) reported that the addition of *Aloe saponaria* extract to conidial suspensions of *C. dematium* f.sp. *epilobii* resulted in a major improvement in disease development. However, efficacy of this viscous adjuvant was only achieved for long dew durations ($\geq$18 h) and was shown to be inconsistent in subsequent studies (data not presented).

In this study, all of the amendments used decreased the above-ground biomass of *E. angustifolium* plants, but only the addition of 25% canola oil to the *C. dematium* f.sp. *epilobii* conidial suspension significantly reduced the above-ground biomass of plants.

Primarily used as chemical herbicide adjuvants (Robinson and Nelson, 1974), the use of oil in bioherbicide preparations, either as an oil emulsion or as an invert-emulsion carrier, has been shown to enhance disease development particularly for plants receiving short dew durations (Quimby *et al.*, 1989; Boyette *et al.*, 1990; Auld, 1993; Yang *et al.*, 1993). In these studies, the vegetable oil emulsion rather than the invert emulsion was chosen based on its lower oil content as well as the high level of phytoxicity on *E. angustifolium* noted with the latter (data not presented).

Several factors have been suggested to be responsible for the increased efficacy with oil emulsion including a facilitation of fungal penetration and establishment (Amsellem *et al.*, 1991) since oil adjuvants are known to modify the integrity of the leaf surface (Hull *et al.*, 1982). Besides causing cuticular damage, some workers have reported that such emulsions decrease the amount of dew required for spore germination and infection on the target weed (Quimby *et al.*, 1989; Daigle *et al.*, 1990; Yang *et al.*, 1993). Similar findings were found in the present study. Even in the absence of dew, disease symptoms were always present, however, the level of control was significantly linked to the oil concentration used. For instance, greater reductions in above-ground biomass were consistently achieved when *C. dematium* f.sp. *epilobii* was applied in combination with 25% canola oil. Some phytotoxicity was observed on oil-treated plant controls, but there was no significant effect of oil alone on the above-ground biomass of *E. angustifolium* seedlings.

The application of *C. dematium* f.sp. *epilobii* conidial suspensions amended with 25% canola oil were beneficial in controlling younger *E. angustifolium* plants by lowering

conidial densities (by a factor of 10). Similar reductions in inoculum threshold with an invert oil emulsion were reported by Amsellem *et al.* (1990).

Acidifying conidial suspensions also increased disease expression and resulted in higher levels of control. The acidic medium might have altered plant physical barriers (Caporn and Hutchinson, 1986) which facilitated fungal foliar penetration. Disease expression was also influenced by the type of buffer used. Within the range of pH tested, lower damage was consistently achieved when the *C. dematium* f.sp. *epilobii* inoculum suspension was buffered with the citrate-phosphate buffer. Gottstein and Kuč (1989) demonstrated that systemic resistance to an anthracnose-causing fungus could be induced by the presence of phosphates. In the present study, the increase in the phosphate proportion of the citrate-phosphate buffer solution at higher pH levels may partly explain the observed inhibitory effect. These results support the findings of Daigle and Cotty (1991) where they demonstrated the influence of both buffer and pH upon the germination and the mycoherbicidal activity of *Alternaria cassia*. Results obtained with the MES-NaOH buffer suggest that disease injury might be increased when *C. dematium* f.sp. *epilobii* is buffered with an alkaline solution. Further studies should therefore examine the effect of such alkaline solutions on *C. dematium* f.sp. *epilobii* disease expression. Also, the effect of pH on dew period requirement should be investigated.

This study has demonstrated the potential of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* seedlings under controlled environment conditions. The oil-based formulation reduced both the exacting environmental requirements of the fungus as well as the inoculum density necessary to achieve a desirable level of control. This formulation

may ensure the mycoherbicidal effectiveness of this fungus under natural conditions, and consequently its potential as an alternative silvicultural management tool and/or complementary strategy to chemical control. The lack of control of mature *E. angustifolium* plants will however restrict the timing of application to the early part of the growing season. Therefore, further research should concentrate on the potential of this bioherbicide for early season applications and to examine ways to improve efficacy on older plants, for example, through a strategy including low rates of chemical herbicides.

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Connecting text

Along with weed control efficacy, host specificity constitutes a key criterion in the development of a fungus as a bioherbicide (Charudattan, 1990)[1]. Although preliminary studies on the host specificity of *C. dematium* f.sp. *epilobii* suggested a restricted host range (Winder and Watson, 1994)[2], more investigation was needed, particularly as to the safety of this potential bioherbicide on tree crops. Hence, this study was conducted with the objectives to expand the previous host range testing and to provide supplemental information regarding the host specificity of *C. dematium* f.sp. *epilobii* concentrating on trees of economic importance in Canada. The susceptibility of tree crops to specific formulations of *C. dematium* f.sp. *epilobii* was also evaluated.

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Host specificity determination of *Colletotrichum dematium* f. sp. *epilobii*, a bioherbicide candidate for the control of *Epilobium angustifolium*

4.1. Abstract

The host range of *Colletotrichum dematium* f.sp. *epilobii*, a potential bioherbicide for the control of *Epilobium angustifolium*, was evaluated on plant species within the order Myrtales and on selected tree species of economic importance in Canadian forestry. In controlled environment studies, *C. dematium* f.sp. *epilobii* was restricted to the Onagraceae family. In addition to *E. angustifolium*, *Camissonia bistorta* and *Clarkia unguiculata* were susceptible to the fungus. *C. unguiculata* is a reported host of a *Colletotrichum* sp., but this is the first report that *C. dematium* is pathogenic to *C. bistorta*. The fungus was highly virulent on all but one ecotype of *E. angustifolium*. All other species tested including nine *Epilobium* species were immune or resistant to *C. dematium* f.sp. *epilobii*. In field studies, yellow birch (*Betula alleghaniensis*) was susceptible to *C. dematium* f.sp. *epilobii* only when conidia were applied in combination with an oil emulsion. The relatively narrow host range of the fungus indicates that *C. dematium* f.sp. *epilobii* could be used selectively in conifer reforestation areas as a biocontrol agent to suppress *E. angustifolium*.

4.2. Introduction

Epilobium angustifolium L. (fireweed), a problematic plant species, is widely distributed throughout the major Canadian reforestation areas. High seed production aids in its ability to rapidly colonize newly disturbed sites (Dyrness, 1973), and its rapid growth reduces the development of newly planted conifer seedlings (Eis, 1981). *E. angustifolium* is commonly controlled with post-emergent applications of chemical herbicides (Gouvernement du Québec, 1991), however, in a context where forest managers face increasing pressures to reduce herbicide use (Walstad, 1988), alternative or complementary control strategies are urgently needed. The use of plant pathogenic fungi as bioherbicides may help to reduce dependence on chemical herbicides for managing undesirable forest vegetation.

In 1988, a foliar necrosis-inducing fungal pathogen, *Colletotrichum dematium* f.sp. *epilobii*, was isolated from diseased *E. angustifolium*, and preliminary studies have demonstrated that this fungus has potential to be further developed as a biocontrol agent for *E. angustifolium* (Winder and Watson, 1994). As part of the development of a bioherbicide, the delimitation of host range is an important factor to consider. Although strict host specificity is not required for native pathogens evaluated as bioherbicides, exhaustive host range studies must be nevertheless conducted to ensure that the candidate agent will not affect non-target plants (Watson, 1985).

C. dematium is the causal agent of diseases found on a broad range of plants that often have a distant phylogenetic relationship. For instance, natural occurrence of *C. dematium* ranges from coniferous trees species, such as *Pinus ponderosa* Douglas ex P.

Laws. & C. Laws (ponderosa pine) to distantly related plant species such as *Fragaria* x *ananassa* Duchesne (cultivated strawberry) or *Lycopersicon esculentum* Miller (tomato) (Anonymous, 1960; Beraha and Wright, 1973; Shaw, 1973). However, prior to the report of Winder and Watson (1994), there was no record of natural occurrence of *C. dematium* on *E. angustifolium*, nor on other species in the *Epilobium* genus.

Although preliminary host range studies to ensure the safety of *C. dematium* f.sp. *epilobii* suggested a restricted host range, more research was required to evaluate precisely the host specificity of this fungus. In this respect, the present study was conducted with the objectives to expand the previous host range testing and to provide supplemental information regarding the host specificity of the *E. angustifolium* isolate of *C. dematium* concentrating on trees of economic importance in Canada. The susceptibility of tree crops to specific formulations of *C. dematium* f.sp. *epilobii* was also evaluated.

4.3. Materials and methods

4.3.1. *Inoculum production.*

Inoculum production was as described in Section 3.3.1 of Chapter 3.

4.3.2. *Host specificity - Controlled environment.*

A total of 23 plant species, representing three families, were grown from seeds obtained from various sources (Table 1). The centrifugal phylogenetic testing sequence proposed by Wapshere (1974) was used as a guide. Several Canadian ecotypes of *E. angustifolium* collected during the summers of 1993 and 1994 were included in this study. Test plant seeds were germinated on moist filter paper, in glass Petri dishes in a dark

incubator at 24 C for one week, transferred to 10-cm-diameter plastic pots containing potting medium (Promix, Premiers Brands Inc., New Rochelle, NY) and thinned to two seedlings per pot following emergence. Seedlings were grown in growth cabinets (24/16 C day/night, 300 $\mu Em^{-2}s^{-1}$, 12 h photoperiod) for four weeks before inoculation. Three pots of each plant species were tested and the experiment was performed twice.

Inoculations were performed on 4-wk-old seedlings at a rate of $1x10^9$ conidia m^{-2} using a spray chamber (Research Instrument Manufacturing Co., Guelph, ON) with a full cone nozzle (TG 0.7), 200 kPa air pressure, a speed of 1 km h^{-1}, and a spray volume of 330 L ha^{-1}. Plants were subsequently placed in a dark dew chamber (Percival Manufacturing Co., Model I-35D, Boone, IA) at 24 C for 24 h, then transferred to a growth chamber having original conditions and arranged in a completely randomized design.

Since disease symptoms caused by the present isolate of *C. dematium* are characterized by rapid although non-expanding lesions, plants were monitored for disease symptoms one week after inoculation. Plants were rated immune (I) if no visible symptoms were observed, hypersensitive (HS) if flecking occurred, resistant (R) if only a few small necrotic lesions occurred, and susceptible (S) if extended necrotic lesions were visible within the 1-wk period. Following rating, lesions were excised from affected plants, surface sterilized in 1.5% NaClO for 15 sec, rinsed in sterile distilled water, and placed on potato dextrose agar (PDA; Difco, Detroit, MI) to confirm that *C. dematium* f.sp. *epilobii* was the causal agent.

4.3.3. *Host specificity - Field.*

Two deciduous trees and seven conifer species were selected for field evaluation

based on their economical importance in Canada. Seedlings of tree species were obtained from various sources (Table 2) and transplanted individually into 22-cm-diameter plastic pots containing potting medium and maintained outside. Pots were fertilized monthly with a 100 ml solution of 20-20-20 fertilizer (3 g L^{-1}) (Plant Products Ltd., Bramalea, ON). Inoculation was performed twice early in September 1993 and 1994 on 1- to 3-yr-old seedlings, when conifer species were hardened. Inoculum was applied in the evening at a rate of $1x10^9$ conidia m^{-2} in a spay volume equivalent to 500 L ha^{-1} using a sprayer equipped with a flat nozzle (Teejet 8003EVS). Three conidial suspensions of *C. dematium* f.sp. *epilobii* were evaluated: a conidial suspension in distilled water, a conidial suspension with 15% canola oil, and a tank mix combination of conidial suspension with 15% canola oil and a low rate of glyphosate (*N*-[phophonomethyl] glycine) (0.1 kg a.i. ha^{-1}). Control trees were sprayed with water only, oil alone, glyphosate alone, and a tank mix combination of oil and glyphosate. The oil formulation was prepared by mixing the canola oil and 0.1% soybean lecithin (w/v) at a high speed in a stainless steel semimicro jar blender (Thomas Scientific, Swedesboro, NJ) for 30 sec. The aqueous phase, containing the conidial suspension, was added to the oil phase and mixed for an additional 30 sec. Treatments including the chemical herbicide were prepared using glyphosate (Roundup®, 356 g L^{-1} of active ingredient). Due to their susceptibility, deciduous species were subjected to all treatments except those containing glyphosate. Each treatment included five trees of each species. For each treatment, 7-wk-old *E. angustifolium* plants, grown originally under greenhouse conditions and transferred outside four weeks after emergence, were also inoculated to serve as susceptible checks. All trees were examined

for disease symptoms for a 3-wk period after inoculation. Disease evaluation followed the rating previously described. As soon as lesions appeared, three foliar lesions were excised from each tree that exhibited disease symptoms, surface sterilized in 1.5% NaClO for 15 sec, rinsed in sterile distilled water, and placed on PDA to confirm whether *C. dematium* f.sp. *epilobii* was the causal agent.

4.4. Results

4.4.1. *Host specificity - Controlled environment.*

Of the 23 plants species inoculated in this study, only three species in the Onagraceae family; *E. angustifolium*, *Camissonia bistorta*, and *Clarkia unguiculata* were susceptible to *C. dematium* f.sp. *epilobii* (Table 1). There were no disease symptoms on species of the families Lythraceae and Myrtaceae. On susceptible species, large irregular tan lesions were mainly limited to leaves and appeared one day following inoculation. No subsequent disease development however was noted within the 1-wk observation period. *Clarkia pulchella* was resistant with symptoms being far less severe, consisting of small necrotic areas and minimal leaf malformation. All ecotypes of *E. angustifolium*, except one, were susceptible to the fungus. Typical necrosis was not observed on leaves of *E. angustifolium* seedlings originating from Oka, Québec, as these plants were hypersensitive to *C. dematium* f.sp. *epilobii*. Visible symptoms were limited to flecks, without subsequent development. Within the genera *Epilobium* and *Oenothera*, all but one of the related plant species were immune to the disease. *Epilobium montanum* was resistant with limited to

Table 1. Selected Myrtales species tested for susceptibility to *Colletotrichum dematium* f.sp. *epilobii*.

Family Genus and species	Source of seed[y]	Reaction[z] Trial 1	 Trial 2
Lythraceae			
Lythrum hyssopifolia L.	Bg & BM	I	I
Lythrum salicaria L.	Bg & BM	I	I
Heimia myrtifolia Cham. & Schlechtensal	Bg & BM	I	I
Myrtaceae			
Leptospermum scoparium Forster & G. Forster	BG & BM	I	I
Onagraceae		I	I
Boisduvalia densiflora (Lindl.) S.Wats.	HbH	I	I
Camissonia bistorta (Torrey & A.Gray) Raven	BG & BM	S	S
Clarkia pulchella Pursh	HbH	R	R
Clarkia unguiculata Lindl.	HbH	S	S
Epilobium alsinifolium L.	MHN	I	I
Epilobium anagallidifolium Lam.	MHN	I	I
Epilobium angustifolium ssp. *angustifolium*	Lac St-Jean,Qc.	S	S
Epilobium angustifolium ssp. *angustifolium*	Rimouski, Qc.	S	S
Epilobium angustifolium ssp. *angustifolium*	Beauce, Qc.	S	S
Epilobium angustifolium ssp. *angustifolium*	Oka, Qc.	-	HS
Epilobium angustifolium ssp. *angustifolium*	Ontario	S	S
Epilobium angustifolium ssp. *albiflorum*	Rimouski, Qc.	S	S
Epilobium ciliatum Raf.	BG & BM	I	I
Epilobium hirsutum L.	BG & BM	I	I
Epilobium hornemannii Rchb.	HbH	I	I
Epilobium komarovianum Lev.	HbH	I	I

Table 1. (continued)

Epilobium montanum L.	HbH	R	R
Epilobium parviflorum Shreb.	JBN	I	I
Epilobium tetragonum L.	HbH	I	I
Gaura parviflora Dougl.	HbH	I	I
Oenothera ammophila Focke	HbH	I	I
Oenothera biennis L.	BG & BM	I	I
Oenothera parviflora L.	HbH	I	I
Oenothera tetragona Roth var. *fraseri* (Pursh) Munz	BG & BM	I	I
Oenothera versicolor Lehm.	HbH	I	I

[y] BG & BM = Botanisher Garten & Botanishes Museum, Berlin-Dahlem; HbH = Hortus botanicus Hauniensis; JBN = Jardin botanique de Nantes, France; MHN = Muséum d'Histoire Naturelle, Paris.

[z] I = immune; HS = hypersensitive; R = resistant; S = susceptible; - = not tested

Table 2. Reaction of selected tree species and *Epilobium angustifolium* to different formulations of *Colletotrichum dematium* f.sp. *epilobii.*

Formulation[x]	Species	Seedling source[y]	Reaction[z] 1993	1994
Cd-W	*Picea glauca* (Moench) Voss (white spruce)	PFB	I	I
	Picea mariana (Mill.) B.S.P. (black spruce)	PFB	I	I
	Pinus strobus L. (white pine)	PFB	I	I
	Pinus resinosa Ait. (red pine)	PFB	I	I
	Betula alleghaniensis Britt. (yellow birch)	PFB	I	I
	Quercus rubra L. (red oak)	PFB	I	I
	Larix laricina (DuRoi) K. Koch (tamarack)	FCM	I	I
	Tsuga heterophylla (Raf.) Sarg. (western hemlock)	PFC	I	I
	Pseudotsuga menziesii (Mirbel) Franco (Douglas fir)	PFC	I	I
	Epilobium angustifolium L. (fireweed)		I	I
Cd-O	*Picea glauca*	PFB	I	I
	Picea mariana	PFB	I	I
	Pinus strobus	PFB	I	I
	Pinus resinosa	PFB	I	I
	Betula alleghaniensis	PFB	S	S
	Quercus rubra	PFB	I	I
	Larix laricina	FCM	I	I
	Tsuga heterophylla	PFC	I	I
	Pseudotsuga menziesii	PFC	I	I
	Epilobium angustifolium		S	S

Table 2. (continued)

Cd-O-G	*Picea glauca*	PFB	I	I
	Picea mariana	PFB	I	I
	Pinus strobus	PFB	I	I
	Pinus resinosa	PFB	I	I
	Larix laricina	FCM	I	I
	Tsuga heterophylla	PFC	I	I
	Pseudotsuga menziesii	PFC	I	I
	Epilobium angustifolium		S	S

[x] Cd-W = conidia + water; Cd-O = conidia + oil; Cd-O-G = conidia + oil + glyphosate

[y] PFB = Pépinière forestière de Berthier, Qc; PFC = Pacific Forestry Center, B.C.; FMC = Forestry Canada - Maritimes, N.B.

[z] I = immune; S = susceptible

small necrotic areas. The fungus was recovered from excised lesions, and microscopic examination of conidia confirmed that the infection was caused by *C. dematium* f.sp. *epilobii*. These results support the findings of Winder and Watson (1994).

4.4.2. *Host specificity - Field.*

Necrotic areas or evidence of disease development were absent from all tree species and susceptible checks inoculated with the unformulated conidia suspension of *C. dematium* f.sp. *epilobii* during the 3-wk observation period (Table 2). Whereas, susceptible checks exhibited dark foliar lesions, and a yellowing and dwarfed aspect with dark foliar lesions following the application of conidial suspensions, with oil and with a tank mix combination of oil and a low rate of glyphosate respectively, no evidence of infection was observed on the conifer species. An oily film was persistent on these species, but no phytotoxic effect was noted. Yellow birch (*Betula alleghaniensis*) was the only tree species susceptible to *C. dematium* f.sp. *epilobii* when conidia were applied in combination with the oil emulsion. Symptoms were first apparent two days following inoculation with irregular dark lesions on leaves. No evidence of phytotoxicity was observed on yellow birch seedlings sprayed with the oil alone. Red oak (*Quercus rubra* L.) was immune to the same treatment. *C. dematium* f.sp. *epilobii* was successfully isolated from excised lesions present on yellow birch. Similar results were obtained when the trial was repeated the following season.

4.5. Discussion

As reported by Watson (1985), host plant specificity of a biocontrol agent

constitutes the most important factor in the decision to develop candidate pathogens as biocontrol agents. The application of Wapshere's strategy (Wapshere, 1974) in this study strongly suggests that *C. dematium* f.sp. *epilobii* is restricted to the family Onagraceae, since no symptoms of infection were found on the tested species belonging to closely related families.

These results confirm, in part, the work of Winder and Watson (1994). In their study, they reported that *C. dematium* was host specific and largely restricted *to E. angustifolium*, although *Epilobium lanceolatum* was identified as susceptible. However, in this study, the susceptibility of *Camissonia bistorta* and *Clarkia unguiculata* of the family Onagraceae to *C. dematium* f.sp. *epilobii*, extend the host range previously determined.

Symptoms of infection caused by *C. dematium* f.sp. *epilobii* on *Camissonia bistorta* and on *Clarkia unguiculata* were equally as severe as those on *E. angustifolium*, consisting of large, but non-expanding areas of necrosis. *C. unguiculata* has been previously reported to be a host of a *Colletotrichum* sp. (Anonymous, 1960). *C. bistorta* and *C. pulchella* have not been previously reported as hosts of *C. dematium* and these findings may be related to conducting the study under controlled environment conditions. As stated by Watson (1985), the accuracy of a host range determination of candidate biocontrol pathogens conducted solely in controlled environment conditions is not adequate. Host range studies performed in such conditions tend to predispose plants to infection which results in artificial expansion of the host range. Nevertheless, the possibility that the pathogen and these species have not come into effective contact in nature or that

the fungus was not observed or reported to occur on these species in natural conditions should also be considered.

The Onagraceae family is of economic importance considering that some species, such as *C. unguiculata* and *C. pulchella*, are sometimes used for ornamental purposes. Testing taxonomically closely related host plants and then extending to other more distantly related taxa (Wapshere, 1974) presupposes that host specificity is closely related to phylogenetic relationship. However, *C. dematium* is the causal agent of diseases found on a broad range of plant species that do not have a close taxonomic relationship. In this study, the occurrence of severe infections on three genera indicate that specificity resides within the Onagraceae family, but these three species are distantly related. Moreover, the absence of many susceptible species within the genus *Epilobium*, other than *E. angustifolium* and *E. lanceolatum* (Winder and Watson, 1994), could also illustrate the lack of a clear relationship between pathogen specificity and phylogenetic relationship.

Of the different ecotypes of *E. angustifolium* tested for susceptibility, individuals originating from a population of the Montréal area near Oka, Québec, were hypersensitive to the fungus. Although seeds of this hypersensitive specimen of *E. angustifolium* originated from a very small population, this finding is nevertheless an indication of the different levels of susceptibility within *E. angustifolium* to *C. dematium* f.sp. *epilobii*. The variability in the response of different ecotypes/biotypes of the same plant species to a pathogen has been previously reported (Hasan, 1972; Hull and Groves, 1973). In this instance, the phenomenon could be related to specific genetic variability present in the

individuals from Oka, since no apparent phenotypic difference was observed at the time of collection.

For economically important tree species growing in locations where *C. dematium* f.sp. *epilobii* may potentially be used as a bioherbicide, none of the tree species tested was found to be affected by the fungus when conidia were applied without any amendment. Unfortunately, susceptible *E. angustifolium* checks remained free of disease when unformulated conidia were used. Consequently, it cannot be confirmed that the absence of disease expression was due to genetic resistance/immunity rather than an inability of *C. dematium* f.sp. *epilobii* to overcome its extended dew period requirement for mycoherbicidal activity (Winder and Watson, 1994; Léger *et al.*, 1995). This is especially evident for *Quercus rubra*, since a related tree, *Quercus kelloggii* Newb. (Kellogg oak) is known to be affected by a related fungus, *C. gloeosporioides* (Anonymous, 1931-1970). Therefore, future studies should investigate the susceptibility of commercially important tree species under optimal conditions for disease expression of *C. dematium* f.sp. *epilobii*.

Both the oil-based formulation of *C. dematium* f.sp. *epilobii* and its tank-mix combination with an oil emulsion and a low rate of glyphosate were included in the present host range testing based on their ability to enhance the effectiveness of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* under controlled environment conditions (Hallett, 1993; Léger *et al.*, 1995). The benefit of oil amendments, either as an emulsion or an invert-emulsion (water-in-oil), as promising carriers for post-emergence spraying of conidial suspension has been extensively reported (Quimby *et al.*, 1989; Boyette *et al.*, 1990; Daigle *et al.*, 1990; Auld, 1993; Yang *et al.*, 1993). Similarly, the reliability of the

strategy combining a fungal pathogen with low rates of chemical herbicides was demonstrated by Wymore and Watson (1989). Of the various tree species tested, only yellow birch (*Betula alleghaniensis*) was found susceptible to *C. dematium* f.sp. *epilobii* when conidia were applied in combination with the oil emulsion. Although yellow birch is not reported to be a host of *C. dematium* or of any related *Colletotrichum* sp., the fungus was successfully re-isolated from diseased leaves. The reduction in selectivity of a mycoherbicidal organism by an oil amendment has been previously reported (Amsellem *et al.*, 1991). Along with decreasing the dew requirement for spore germination and infection (Quimby *et al.*, 1989; Auld, 1993; Yang *et al.*, 1993), it is known that such adjuvants may affect the leaf cuticle (Hull *et al.*, 1982; McWorther and Barrentine, 1988) and facilitate fungal penetration and establishment (Amsellem *et al.*, 1991), although no visible foliar damage was apparent with the oil controls of yellow birch. In addition, the phenomenon of predisposition could explain the observed susceptibility of this tree species to the oil-based formulation of *C. dematium* f.sp. *epilobii*. Since this host specificity test was conducted at the end of the growing season, deciduous trees were probably starting to senesce, rendering them more susceptible to the action of the fungus. Consequently, it can be hypothesized that yellow birch would have exhibited a different response if host-range testing would have been conducted earlier in the season.

Under controlled environment, the *C. dematium* f.sp. *epilobii* host range is apparently limited to the Onagraceae family. Since tree species inoculated with different formulations of the fungus remained free of disease symptoms under field conditions, with the exception of yellow birch, this fungus could be selectively used as a silvicide agent to

control *E. angustifolium* plants. However, field trials have demonstrated that optimal control is achieved only when a formulation including low rate of glyphosate is applied on younger *E. angustifolium* plants (Léger *et al.*, 1995). Therefore, additional host range studies should investigate the susceptibility of commercial tree species during the early stage of the growing season, when most conifer species are sensitive to chemical herbicide applications.

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Connecting text

Once a solid understanding of both efficacy and safety aspects is met under controlled environment conditions, the reliability of the bioherbicide candidate should be ascertained under field conditions. As illustrated in previous studies, both the exacting environmental requirements of *C. dematium* f.sp. *epilobii* and its weak mycoherbicidal activity on mature plants could strongly limit its effectiveness to control *E. angustifolium* under natural conditions. In this respect, small scale field trials were conducted with the purpose of evaluating the benefit of suitable formulations to ensure field efficacy of *C. dematium* f.sp. *epilobii*, particularly against more mature plants.

Field efficacy of formulated spore suspensions of *Colletotrichum dematium* f. sp. *epilobii* for the control of *Epilobium angustifolium*

5.1. Abstract

Field effectiveness of some formulations of the fungal pathogen *Colletotrichum dematium* f.sp. *epilobii* to control *Epilobium angustifolium*, a major weed species in coniferous regeneration areas, is reported. Where *C. dematium* f.sp. *epilobii* alone or in an oil emulsion failed to control *E. angustifolium* growth, the application of both components in a tank mix combination with a low rate of glyphosate constantly provided the highest level of control. Growth reductions achieved with this formulation were more than additive, suggesting a synergistic interaction. The effectiveness of formulated conidial suspensions of *C. dematium* f.sp. *epilobii* significantly decreased with plant maturity. Results were fairly constant over the two growing seasons. If properly formulated and strategically applied during the growing season, *C. dematium* f.sp. *epilobii* can provide some suppression of *E. angustifolium.*

5.2. Introduction

Throughout the major Canadian reforestation areas, *Epilobium angustifolium* L. (fireweed) is a troublesome perennial weed species. With its ability to rapidly colonize

newly disturbed sites, this forest weed species is able to reduce the growth of newly planted conifer seedlings (Dyrness, 1973; Eis, 1981).

Although several silvicultural practices are available for controlling undesirable vegetation, new methods are especially needed for *E. angustifolium*. Some silvicultural practices, such as prescribed burning or mechanical clearing, reduce competition from associated species and provide a suitable seedbed leading to the establishment and development of *E. angustifolium* (Bartos and Mueggler, 1981). Chemical control, although effective, can be problematic, particularly during the hardening-off period when young conifer seedlings are most sensitive to chemical herbicides. Moreover, projected increases in the worldwide demand for wood products put added pressure on forest managers to intensify reforestation success, in light of increasing pressure in North America to reduce herbicide use (Walstad, 1988; Wagner and Zasada, 1991). In this context, the use of plant pathogenic agents as a silvicultural tool should be considered. This approach, commonly refered to as the bioherbicide strategy, involves the application of indigenous fungal plant pathogens in a manner analogous to chemical herbicides. Although major advancements have been underway in the agricultural sector, several projects are being conducted in Canada for the control of forest weed species (Watson and Wall, 1995).

In 1988, a foliar pathogen, *Colletotrichum dematium* f.sp. *epilobii*, was isolated from *E. angustifolium* collected near Rimouski, Québec. Although preliminary studies have demonstrated its potential as a bioherbicide, the need for prolonged leaf wetness periods for successful disease establishment and the lack of control of older plants severely

limits its reliability to control *E. angustifolium* under field conditions (Winder and Watson, 1994).

This study dealt with the evaluation of formulated conidial suspensions of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* under natural conditions. The objective was to determine if inoculum suspensions of *C. dematium* f.sp. *epilobii* amended with an oil emulsion or as a tank mix with the oil emulsion and a low dose of glyphosate (*N*-[phophonomethyl]glycine) would improve field efficacy of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* seedlings and mature plants.

5.3. Material and methods

5.3.1. *General procedures.*

Field trials were carried out in 1993 and 1994, on a St-Bernard loam (orthic melanic brunisol) at the Horticultural Research Center, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Québec. The experiment was set-up as a randomized complete block design, with four plots for each treatment, and four blocks arranged so as to compensate for the potential shade effect of an adjacent sorghum field. The experimental site was kept weed-free by hand-weeding throughout the growing season. Daily minimum and maximum temperatures and precipitation were provided by the Service de la météorologie, Ministère de l'Environnement du Québec, for the Ste-Anne-de-Bellevue station, located approximately 2 km from the experimental site.

5.3.2. *Plant and inoculum production.*

E. angustifolium seeds collected from a natural population in the Beauce region of

Québec were sown in plastic trays filled with potting medium (Promix, Premiers Brands, New Rochelle, NY), and placed in the greenhouse at 24 ± 4 C with no supplemental lighting. Two weeks following seedling emergence, trays were transferred outside for two weeks for acclimatization of the seedlings. Four-wk-old *E. angustifolium* plants were transplanted in the field, and watered daily.

C. dematium f.sp. *epilobii* was originally isolated from diseased *E. angustifolium* plants collected in the Rimouski area of Québec in 1988 and stock cultures were maintained at 4 C on potato dextrose agar (PDA; Difco, Detroit, MI) slants (Winder and Watson, 1994). Inoculum was produced according to the methodology described in Section 3.3.1 of Chapter 3.

Treatments containing an oil emulsion were prepared by mixing the desired volume of canola oil with 0.1% soybean lecithin (w/v) at high speed for 30 sec in a stainless steel semimicro jar blender (Thomas Scientific, Swedesboro, NJ). The aqueous phase, containing the spore suspension, was added to the oil phase and mixed for an additional 30 sec. Treatments including a chemical herbicide were prepared using glyphosate (Roundup®, 356 g L^{-1} of active ingredient).

5.3.3. *Application procedure and weed control assessment.*

Treatments were applied in the evening, with an inoculum density of $1x10^9$ conidia m^{-2} using a sprayer equipped with a flat nozzle (Teejet 8003EVS) with a spray volume equivalent to 500 L ha^{-1}. A portable plastic shield was used around each plot during spraying time to minimize spray drift. Plants were harvested three weeks after treatment by

cutting plants at the soil level. The height (cutting line to the highest point) was recorded for each plant, and above-ground biomass dried for 24 h at 70 C, and weighed.

5.3.4. *1993 trial.*

Each plot (1 x 1m) contained 25 *E. angustifolium* seedlings, and were arranged in a square grid 1.5 m apart. *C. dematium* f.sp. *epilobii* conidial suspensions were applied either in distilled water, in 15% (v/v) of canola oil, or in a tank mix combination with 15 % of canola oil and 0.1 kg a.i. ha^{-1} glyphosate. Control plots were sprayed with water only, glyphosate alone, and a tank mix combination of oil and glyphosate. Treatments were applied on 5 August on 7-wk-old plants. The nine plants positioned in the central part of each plot were harvested in order to minimize edge effect.

5.3.5. *1994 trial.*

Each plot (0.75 x 0.75 m) consisted of nine *E. angustifolium* plants, and were arranged in a square grid 1.5 m apart. Based on the lack of control observed in 1993, the *C. dematium* f.sp. *epilobii* in distilled water treatment was not evaluated during this growing season. Treatments consisted of conidial suspensions of *C. dematium* f.sp. *epilobii* amended with 25% (v/v) canola oil and a tank mix combination of the oil-based formulation and 0.1 kg a.i. ha^{-1} glyphosate. Control plots were sprayed with distilled water only, the oil emulsion only, glyphosate alone, and with a tank mix combination of oil emulsion and glyphosate. Treatments were applied on 15 June on 7-wk-old plants and the experiment was repeated on 15 August on a separate set of 15-wk-old plants.

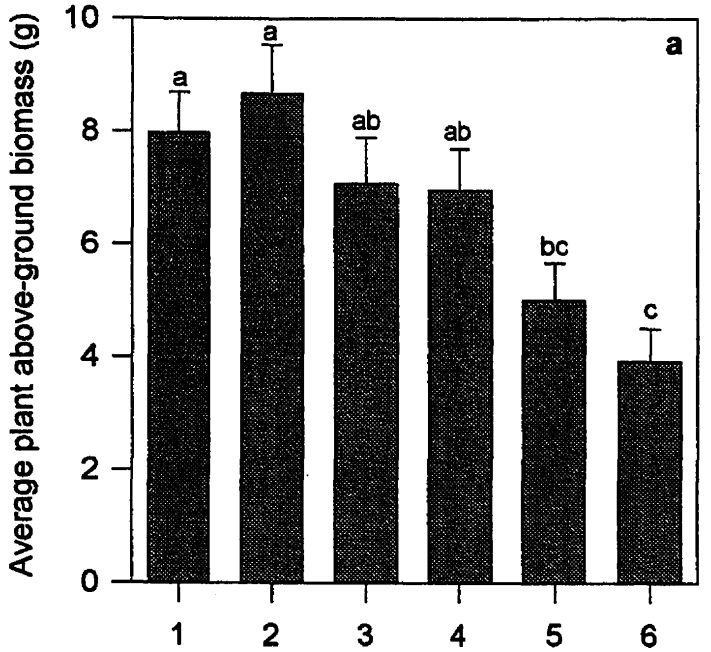
5.3.6. *Data analysis.*

All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Data from experiments were subjected to analysis of variance (GLM procedure) and treatment means compared using the Student-Newman-Keuls Multiple Range test (SNK) at $P = 0.05$. Logarithmic transformation [log (x + 1)] was performed to obtain homogeneity of variance.

5.4. Results

Formulations greatly influenced the ability of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* growth under field conditions. In the 1993 field study (Fig. 1), the application of *C. dematium* f.sp. *epilobii* alone did not reduce either the height or the above-ground biomass of *E. angustifolium* seedlings. Similarly, typical tan lesions produced by the fungus in controlled environment studies were absent during the course of this experiment. The oil-based formulation (15% canola oil) of *C. dematium* f.sp. *epilobii* provided a slight reduction of both above-ground biomass (11.3%) and height (3.1%) of 7-wk-old *E. angustifolium* plants. The highest level of control was obtained when the oil-based formulation was applied in a tank mix combination with glyphosate at 0.1 kg a.i. ha^{-1}. Above-ground biomass and height were reduced by 21.6% and 21.2% as compared with the corresponding control treatment. However, presumably due to the large variability observed in the field, mean above-ground biomass of plants subjected to the tank-mix application was not significantly different from the oil/glyphosate control treatments. Plant

Figure 1. Effect of various *Colletotrichum dematium* f.sp. *epilobii* formulations on (**a**) above-ground biomass and (**b**) height of *Epilobium angustifolium* plants under field conditions in 1993. Treatment 1 = water; 2 = conidia + water; 3 = conidia + oil (15% v/v); 4 = glyphosate (0.1 kg a.i. ha^{-1}); 5 = oil (15% v/v) + glyphosate (0.1 kg a.i. ha^{-1}); and 6 = conidia + oil (15% v/v) + glyphosate (0.1 kg a.i. ha^{-1}). Inoculations were performed on 7-wk-old plants at a density of $1x10^9$ conidia m^{-2}. Columns having the same letter are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.


Average plant above-ground biomass (g)
10
8
6
4
2
0
a
a
ab
ab
bc
c
a
1
2
3
4
5
6

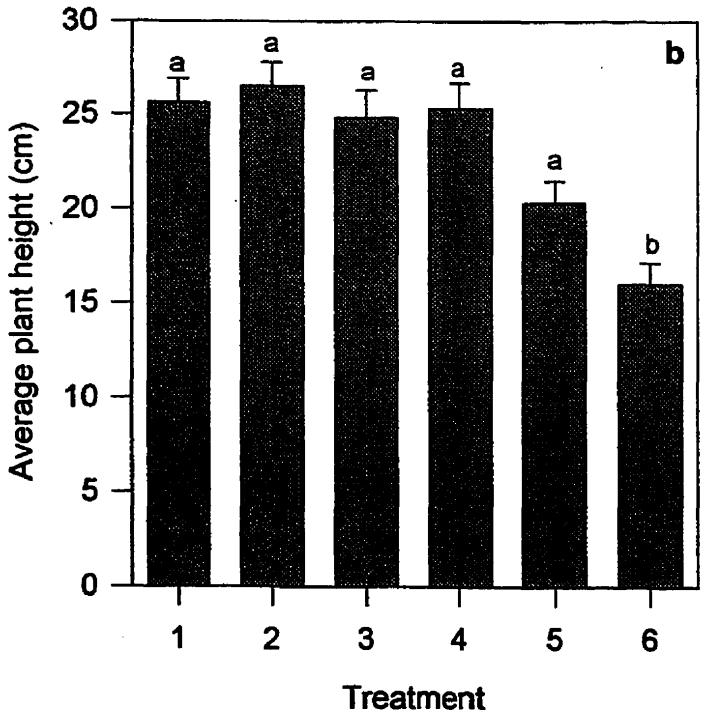
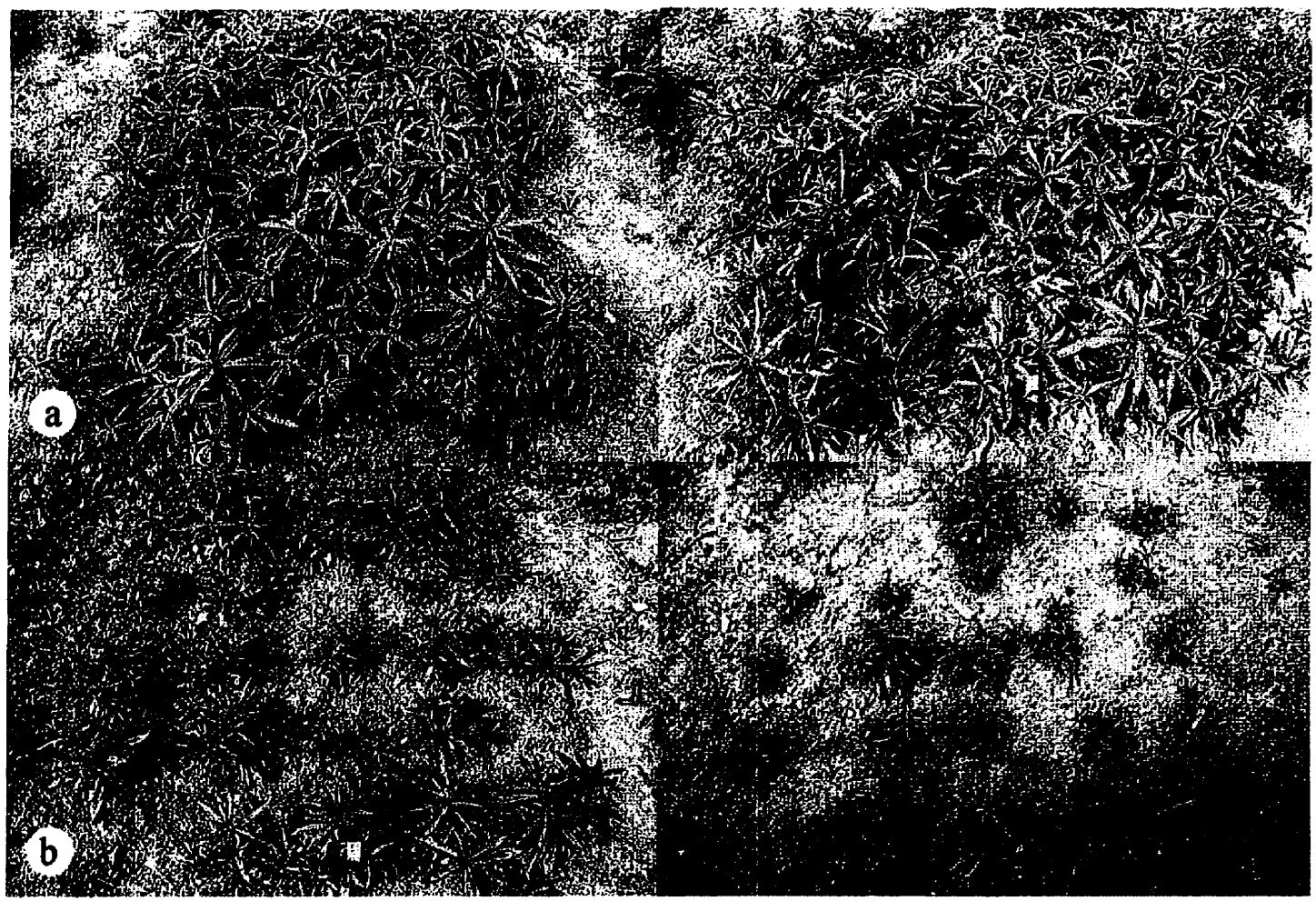

Average plant height (cm)
30
25
20
15
10
5
0
a
a
a
a
a
b
b
1
2
3
4
5
6
Treatment

Figure 2. Effect of *Colletotrichum dematium* f.sp. *epilobii* inoculum suspensions applied (**a**) alone or in (**b**) a tank-mix combination with canola oil (15% v/v) and glyphosate (0.1 kg a.i. ha^{-1}) to 7-wk-old *Epilobium angustifolium* plants under field conditions in 1993. Inoculation was performed using a density of $1x10^9$ conidia m^{-2}. Respective control treatments are on the left. Pictures were taken three weeks following treatment.

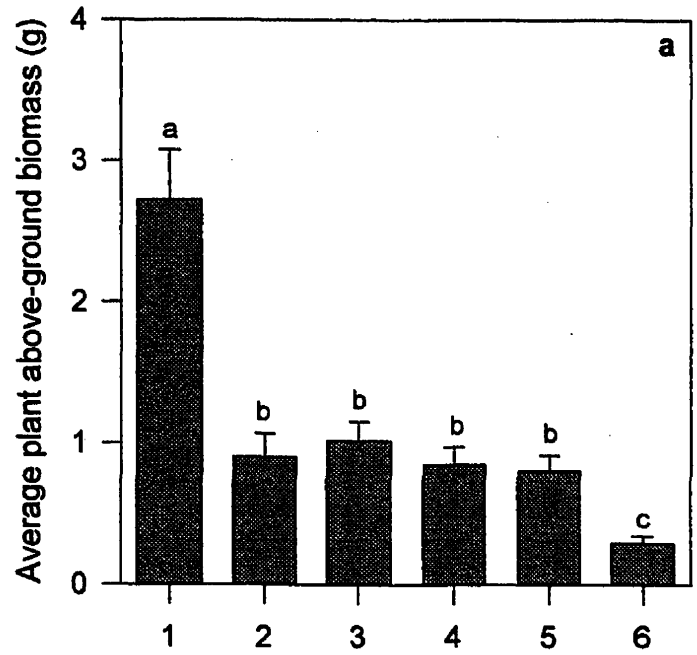

a
b

morphology was considerably affected when using this formulation as dwarfing and yellowing were observed (Fig. 2).

Based on the lack of control observed for both the unformulated conidial suspension of *C. dematium* f.sp. *epilobii* and its oil-based formulation in 1993, the 1994 field experiment was slightly modified by deleting the conidia in water treatment from subsequent evaluation, and by increasing the oil concentration up to 25%. However, despite the significant level of damage caused by the oil-based formulation in comparison with water control plants, both above-ground biomass and height were not significantly different in the corresponding measurements for the oil control treatment (Fig. 3). The application of the oil emulsion alone at 25% gave major phytotoxic effects, expressed as leaf burning in the upper portions of the plants. Similar to the 1993 field experiment, the tank-mix application of the formulated oil-based suspension of *C. dematium* f.sp. *epilobii* with a low rate of glyphosate provided the highest level of control in 1994, which was significantly greater than the control obtained from the oil/glyphosate control treatments or their components alone. Hence, above-ground biomass and height were reduced by 63.6% and 38.4%, respectively, compared with the oil/glyphosate control treatments.

In contrast with younger *E. angustifolium* plants, all of the treatments evaluated in 1994 failed to provide significant reduction in growth of more mature plants (15-wk-old), causing minimal reductions in above-ground biomass and height (Fig. 4). The pattern of growth reduction for the different treatments was fairly consistent with the experiment using younger plants and the highest level of control of more mature plants was achieved

Figure 3. Effect of various *Colletotrichum dematium* f.sp. *epilobii* formulations on (**a**) above-ground biomass and (**b**) height of 7-wk-old *Epilobium angustifolium* plants under field conditions in 1994. Treatment 1 = water; 2 = oil (25% v/v); 3 = conidia + oil (25% v/v); 4 = glyphosate (0.1 kg a.i. ha^{-1}); 5 = oil (25% v/v) + glyphosate (0.1 kg a.i. ha^{-1}); and 6 = conidia + oil (25% v/v) + glyphosate (0.1 kg a.i. ha^{-1}). Inoculations were performed at a density of $1x10^9$ conidia m^{-2}. Columns having the same letter are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.


a
Average plant above-ground biomass (g)
4
3
2
1
0
a
b
b
b
b
c
1
2
3
4
5
6

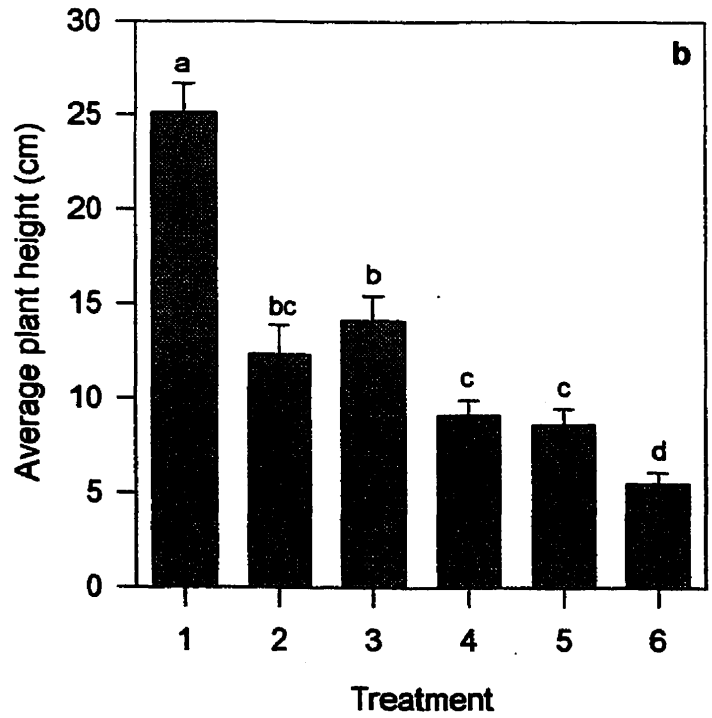
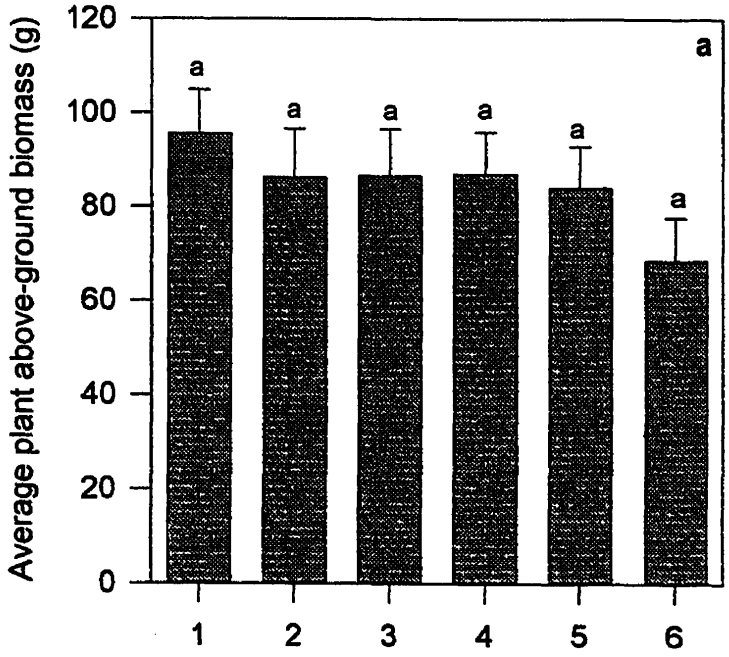

b
Average plant height (cm)
30
25
20
15
10
5
0
a
bc
b
c
c
d
1
2
3
4
5
6
Treatment

Figure 4. Effect of various *Colletotrichum dematium* f.sp. *epilobii* formulations on **(a)** above-ground biomass and **(b)** height of 15-wk-old *Epilobium angustifolium* plants under field conditions in 1994. Treatment 1 = water; 2 = oil (25% v/v); 3 = conidia + oil (25% v/v); 4 = glyphosate (0.1 kg a.i. ha^{-1}); 5 = oil (25% v/v) + glyphosate (0.1 kg a.i. ha^{-1}); and 6 = conidia + oil (25% v/v) + glyphosate (0.1 kg a.i. ha^{-1}). Inoculations were performed at a density of $1x10^9$ conidia m^{-2}. Columns having the same letter are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.


Average plant above-ground biomass (g)
120
100
80
60
40
20
0
a
a
a
a
a
a
a
1
2
3
4
5
6

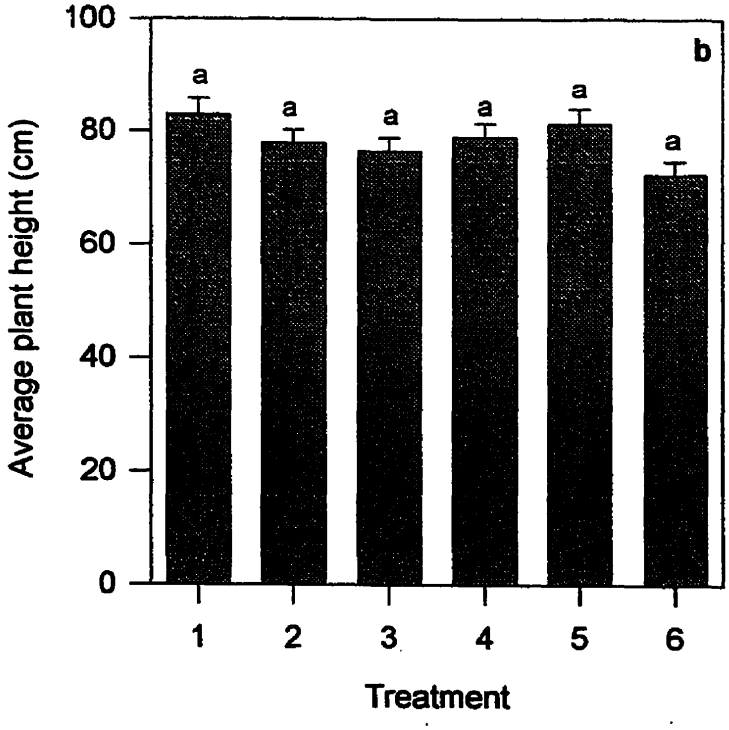

Average plant height (cm)
100
80
60
40
20
0
a
a
a
a
a
a
b
1
2
3
4
5
6
Treatment

Figure 5. Daily maximum (———) and minimum (– – – –) temperature (C) and precipitation (mm) (bars) following inoculation in 1993. Treatment date is indicated by the arrow.

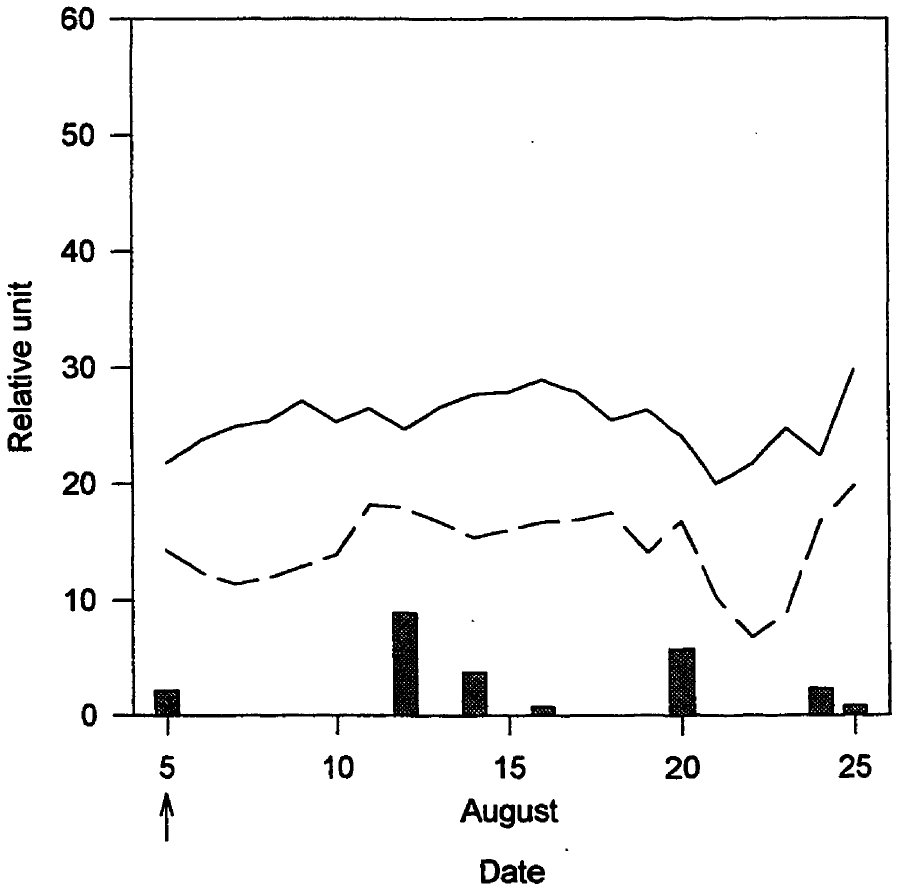
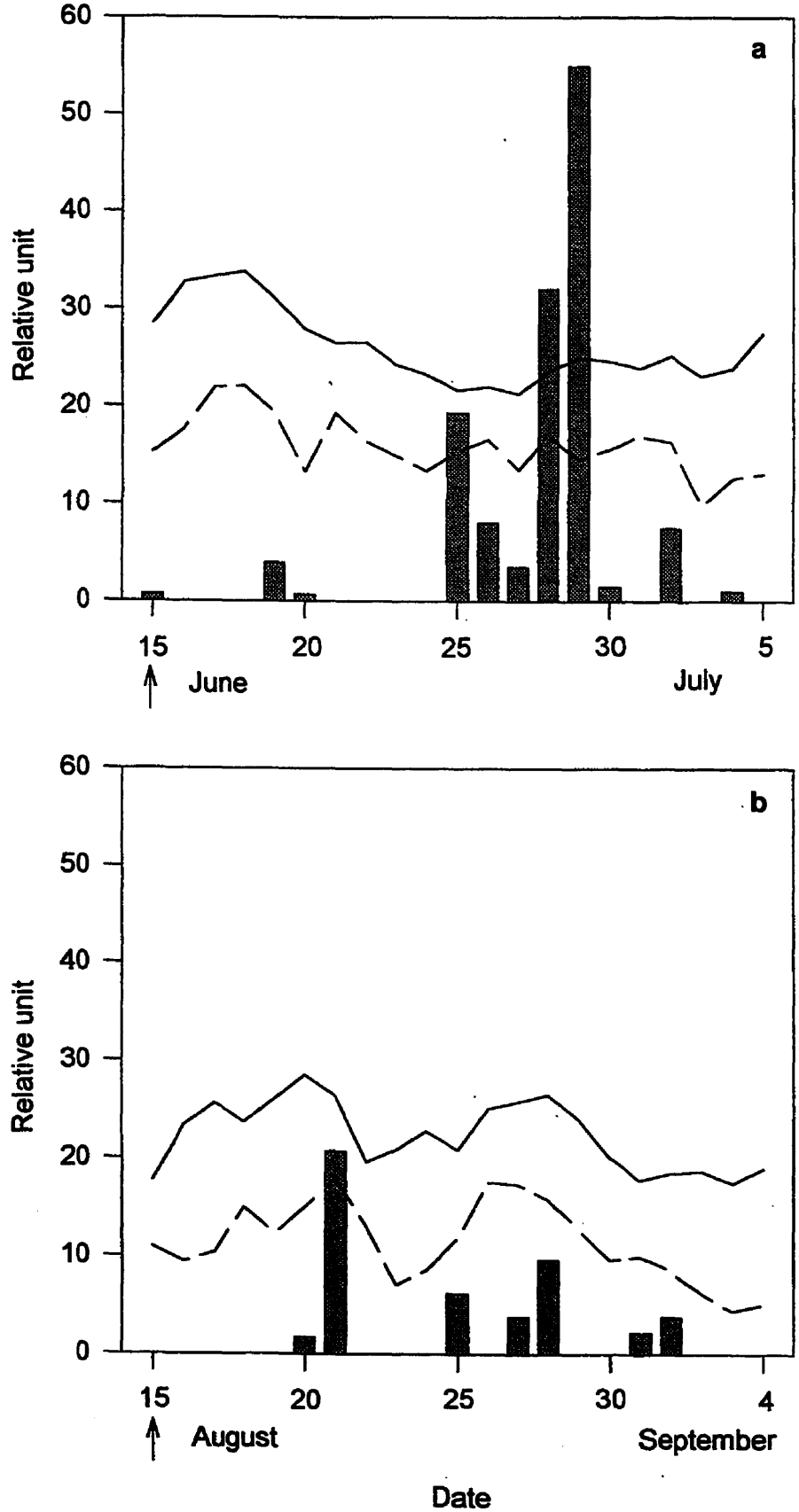

60
50
40
30
20
10
0
Relative unit
5
10
15
20
25
August
Date

Figure 6. Daily maximum (———) and minimum (– – – –) temperature (C) and precipitation (mm) (bars) following inoculations of (**a**) 7-wk-old plants and (**b**) 15-wk-old *Epilobium angustifolium* plants in 1994. Treatment date is indicated by the arrow.


a
b
Relative unit
60
50
40
30
20
10
0
15
20
25
30
5
4
June
July
August
September
Date

by using the tank mix preparation of the oil-based formulation of the fungus with a low rate of the herbicide.

Conditions were dry at the time of treatment application in both 1993 and 1994. In 1993, rain occurred only seven days following inoculation, when 8.9 mm fell on 12 August, with a total precipitation of 24.3 mm for the entire observation period (Fig. 5). In 1994, showers occurred four days following the inoculation of 7-wk-old plants, when 3.9 mm fell on 19 June (Fig. 6). For 15-wk-old treated plants, rain did not occur until five days after inoculation when 1.7 mm fell on 20 August (Fig. 6). Total precipitation was 133 mm and 48 mm from 15 June to 6 July and from 15 August to 5 September, respectively. In 1993, the average daily maximum and minimum temperatures from 5 August to 26 August were 25.4 and 14.6 C, respectively. In 1994, the average daily maximum and minimum temperatures from 15 June to July 6 were 26.2 and 16 C, respectively, and 22.3 and 11.4 C, respectively, from 15 August to 5 September.

5.5. Discussion

As living organisms, bioherbicides are sensitive to environmental conditions with free water and temperature greatly influencing the mycoherbicidal activity of these pathogens. Therefore, the failure to control *E. angustifolium* seedling growth during the 1993 field trial when *C. dematium* f.sp. *epilobii* conidia were applied alone was somewhat expected. Controlled environment studies indicated that rather exacting environmental conditions would affect the reliability of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* under natural conditions (Chapter 3). The night temperatures that followed

immediately after inoculation, with a minimum of 12.4 C, may have inhibited disease development, and the total precipitation of 24 mm during the entire period of observation severely restricted disease development (Fig. 5). This result with the unformulated conidial suspension of *C. dematium* f.sp. *epilobii* support the findings of a field trial carried out in 1992 (data not presented).

Undoubtedly, the development of suitable formulations represents one of the foremost considerations when using the bioherbicide biocontrol strategy. Numerous studies have demonstrated the utilization of fungal formulations to compensate for exacting environmental requirements of bioherbicide pathogens. For instance, the use of oil, either as an emulsion or as a water-in-oil (invert) emulsion, has been shown to reduce and even to overcome, dew dependence of some plant pathogenic fungi (Quimby *et al.*, 1989; Daigle *et al.*, 1990; Auld, 1993; Yang *et al.*, 1993). In spite of promising results in helping to induce severe disease damage to young plants at lower dew durations and temperatures in controlled environment studies (Chapter 3), the benefits of the oil-based formulation of *C. dematium* f.sp. *epilobii* during the course of the field trials were not apparent. For instance, during 1994 field testing, both the conidial suspension of *C. dematium* f.sp. *epilobii* formulated in the oil-based emulsion and the respective treatment control reduced *E. angustifolium* growth to a similar level. Since both moisture and temperature conditions that occurred during the period following the inoculation of 7-wk-old plants in 1994 were probably not limiting for disease development of the oil-based formulation of *C. dematium* (Fig. 6), the induced phytotoxicity at a degree much higher

than that was previously observed in controlled environment studies could have contributed to the lack of control.

Several examples have illustrated the reliability of the strategy integrating a fungal pathogen with low rates of chemical herbicides to control a target weed, and this approach was recently subjected to a review by Altman *et al.* (1990). In this study, the oil-based formulation in a tank mix combination with glyphosate, at about 20 times less than the recommended rate, displayed considerable promise as a formulation for *C. dematium* f.sp. *epilobii* to control *E. angustifolium* under natural conditions. In both 1993 and 1994, the highest level of control was achieved with this formulation. The fact that the level of control sustained by the tank mix combination was much higher than the respective treatment control or its component alone might suggest a synergistic interaction. These results are supported by previous controlled environment studies on synergism (Hallett, 1993) as well as conclusions of the work of Wymore and Watson (1989).

The precise role of chemical herbicides in inducing disease development is not well understood, but it is proposed that low doses of chemical herbicides impose additional stress on the plant in a manner that such weed hosts could be more susceptible to infection by a plant pathogen resulting in more effective weed control (Altman *et al.*, 1990). Sharon *et al.* (1992) reported the increased susceptibility of a weed to a mycoherbicide when subjected to glyphosate. By its action on the shikimic acid pathway, glyphosate has a direct influence upon the synthesis of phenolic compounds, which are known to be involved in plant defense mechanisms (Kosuge, 1969; Friend, 1979). It is possibly for this reason that

sublethal rates of glyphosate are able to predispose *E. angustifolium* by *C. dematium* f.sp. *epilobii* infection.

Plant age was found to play a major role in the efficacy of the different formulations of *C. dematium* f.sp. *epilobii* to control *E. angustifolium* growth. In 1994, none of the treatments evaluated showed significant impact on the growth of 15-wk-old plants, although the highest level of control was achieved with the application of the oil-based emulsion in a tank mix combination with glyphosate. As hypothesized by Winder and Watson (1994), the period after inoculation was probably too short for the production of weight differences which could be separated from variance in the field. Nevertheless, these results are somewhat similar to those obtained in laboratory studies in which a negative relationship was established between the severity of the disease caused by *C. dematium* f.sp. *epilobii* and the age of *E. angustifolium* plants (Chapter 3). In addition, it can be presumed that environmental conditions, with average temperatures of 5 C and total precipitation of 48 mm, which were lower than for the period that followed the inoculation of 7-wk-old plants during the same growing season, would have restricted the mycoherbicidal activity of *C. dematium* f.sp. *epilobii*.

Results in these experiments demonstrate that under natural conditions, *E. angustifolium* growth can be controlled when *C. dematium* f.sp. *epilobii* is properly formulated and strategically applied during the growing season. The addition of a low rate of glyphosate provides some interesting ways for future development of *C. dematium* f.sp. *epilobii* as silvicultural tool for the control of *E. angustifolium*. Since the rapid regeneration from rhizomes is an important issue in dealing with the control of *E.*

angustifolium using *C. dematium* f.sp. *epilobii* (Winder and Watson, 1994), glyphosate could provide substantial benefits solely over the fungus by its ability to translocate to underground propagules of this perennial species.

In a context where vegetation management practices in forestry are progressively directed in a manner similar to the integrated pest management (IPM) concept developed in agriculture (Wagner and Zasada, 1991), the bioherbicide biocontrol strategy has a possible viable niche. The benefits in integrating this strategy with existing silvicultural control measures, particularly chemical methods, would assist in reducing levels of chemical herbicides in the environment.

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Connecting text

While the research comprising this thesis focused on the assessment of *Colletotrichum dematium* f.sp. *epilobii* to control *Epilobium angustifolium* and has been described in the previous chapters, efforts were concurrently devoted to the investigation of other potential bioherbicides for the control of *E. angustifolium*. Recently, an *Alternaria* sp. was isolated from a foliar lesion on *E. angustifolium*, collected north of Québec City. Studies indicated that it had significant bioherbicide potential, however, later investigations failed to provide consistent results when aqueous conidial suspensions of this isolate were used. Hence, the potential of this bioherbicide candidate was subsequently evaluated using a new formulation consisting of an oil-based emulsion of ground and colonized solid substrate of the fungus. Initial results are discussed in this chapter.

Chapter 6

Evaluation of a new technique of formulating a biocontrol agent candidate for the control of *Epilobium angustifolium*

6.1. Abstract

Results of a new technique of formulating a bioherbicide candidate for the control of *Epilobium angustifolium*, a problematic weed species within the Canadian reforestation sector, are reported. An *Alternaria* isolate was grown on wheat seeds, and the culture dried, ground, and sieved to produce a powder containing $1x10^6$ conidia g^{-1}. The dried powder was suspended into a solution containing a mixture of a vegetable oil emulsion (11.1% v/v) and gelatin (1% w/v), and applied at a rate of 50 kg ha^{-1} in a volume of 450 L ha^{-1}. Under controlled conditions, post-emergence application induced severe foliar and stem lesions of *E. angustifolium* plants. This formulation significantly reduced above-ground biomass of *E. angustifolium* seedlings when applied at a rate as low as $5x10^6$ conidia m^{-2} with a 12-h dew. The observed mycelial growth following dew incubation suggests that this formulation provides supplemental nutrients that may have favoured vegetative growth rather than conidia formation, germination, and penetration. Formulation including longer-aged colonized culture material could, therefore, exhibit greater virulence. Similar results were obtained using this formulation method with *Colletotrichum dematium* f.sp. *epilobii*, another bioherbicide candidate of *E.*

angustifolium. These results demonstrate the potential of this formulation and suggest that it could be extended to other microbial biocontrol agents.

6.2. Introduction

Within Canadian reforestation areas, *Epilobium angustifolium* L. (fireweed) is a major troublesome perennial weed species. This tall herb which reproduces both by seeds and underground horizontal roots is commonly found in the early phases of forest regeneration. With its ability to rapidly colonize newly disturbed sites, *E. angustifolium* reduces the growth of newly planted conifer seedlings (Dyrness, 1973; Eis, 1981).

Despite the common use of chemical herbicides to control *E. angustifolium*, alternative or complementary control strategies are urgently needed given that forest managers face increasing pressure to reduce chemical herbicide use (Walstad, 1988), and even a total ban in Québec (Gouvernement du Québec, 1991).

The use of plant pathogenic fungi as bioherbicides may help to reduce dependence on chemical herbicides for managing undesirable forest vegetation. Most of the bioherbicide candidates are applied as liquid suspension, which are best suited for use as post-emergence to incite leaf and stem diseases (Boyette *et al.*, 1991). However, short shelf life of such liquid-based bioherbicides can limit their practical use. In this respect, solid-based bioherbicide candidates, such as *Fusarium oxysporum* var. *cannabis* Snyd. and *Alternaria macrospora* Zimm., or liquid-based bioherbicides that include a rehydratable active component, such as *Colletotrichum gloesporioides* f.sp. *aeschynomene*, are often viewed as more stable (Boyette *et al.*, 1991). Recently, an improved formulation of a

biocontrol candidate of *E. angustifolium*, *Alternaria* sp., consisting of a mixture of the ground, colonized solid substrate and an oil emulsion, was developed. The objective of this study was to evaluate the efficacy of this formulation to control *E. angustifolium*.

6.3. Materials and methods

6.3.1. *Inoculum production*.

The *Alternaria* isolate was originally isolated from a diseased *E. angustifolium* specimen collected in the Parc des Laurentides, north of Québec City, Québec in 1994. Diseased leaves were first surface sterilized in 70% ethanol for 2-3 sec, in 1.5% NaClO for 15 sec, and rinsed in sterile distilled water. Lesions were aseptically excised (5 mm^2) and plated on half strength potato dextrose agar (1/2 PDA; Difco, Detroit, MI) containing novobiocin at 150 mg L^{-1}. Cultures were incubated at 24 C for two days, after which the fungus was isolated using the hyphal tip technique. Stock cultures were maintained at 4 C on PDA slants. Starter cultures were prepared by aseptically transferring small pieces of mycelium from stock cultures to PDA plates and inoculated plates kept at room temperature (21 ± 1 C) for two weeks.

Twenty grams of wheat seeds were placed in 250 ml Erlenmeyer flasks, moistened with 20 ml of distilled water, and autoclaved for 17 min at 100 kPa and 120 C. Two mycelial agar plugs (6-mm-diameter) were removed from the leading edges of 14-day-old starter colonies of *Alternaria* sp. and used to inoculate each flask of wheat seed. The flasks were incubated for 14 days at room temperature (21 ± 1 C) under 12 h near ultra-violet light (NUV; J-05, UVP, Inc., Circleville, OH), and shaken every day to prevent

substrate aggregation. The culture material was dried for two days at room temperature, finely grounded for 1 min using a coffee grinder, and sieved. The resulting powder (250 to 425 μm) contained approximately $1x10^6$ conidia g^{-1}. An oil emulsion was prepared by stirring canola oil (11.1% v/v) and 0.4% soybean lecithin (w/v) in distilled water at a high speed for 30 sec in a stainless steel semimicro jar blender (Thomas Scientific, Swedesboro, NJ). Five grams of the colonized, ground wheat substrate and 1% gelatin (w/v) were added to the oil phase and mixed for an additional 30 sec.

6.3.2. *Plant production*.

E. angustifolium seed was collected in 1995 from a population in the Beauce region of Québec, and plants produced according to the methodology described in Section 3.3.2. of Chapter 3.

6.3.3. *Inoculation procedures and weed control assessment*.

The ground wheat suspended formulation was applied on 5-wk-old seedlings using a spray chamber (Research Instrument Manufacturing Co. LTD, Guelph, ON), 200 kPa air pressure and a speed of 1 km h^{-1}. Due to the high viscosity of the formulation, the volume of the formulation was 450 L ha^{-1} and was applied through a flat nozzle (Teejet 8004EVS) at a rate of $5x10^6$ conidia m^{-2}. Plants were subsequently placed in a dark dew chamber (Percival Manufacturing Co., Model I-35D, Boone, IA) at 21 C for 12 h, then transferred to a growth chamber with the original conditions. Control treatments consisted of spraying plants with a "blank" formulation without the fungus or with distilled water only .

Disease severity was estimated one week after inoculation using a rating system based on a scale from 0 to 4 where 0 = no visible symptom and 4 = > 75% necrosis. Three weeks following inoculation, *E. angustifolium* plants were harvested. Live plants were cut at the soil line and above-ground biomass was determined by drying aerial tissue for three days at 60 C, and weighing. Due to the difficulty of excising healthy areas accurately from diseased tissues, weights included the whole aerial portion of the plants.

6.3.4. *Data analysis*.

All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Experiments were performed twice using a completely randomized design with five replicates per treatment. Data from experiments were subjected to analysis of variance (GLM procedure) and treatment means compared using the Student-Newman-Keuls Multiple Range test (SNK) at $P = 0.05$. Logarithmic transformation of raw data [log (x + 1)] was performed to obtain homogeneity of variance. Variance between the two trials of each experiment was compared by using the Chi-square test for homogeneity of variance (Gomez and Gomez, 1984). Variances were homogeneous and results from the two experiments were pooled.

6.4. Results

Application of the oil-based formulation which included the colonized substrate of *Alternaria* sp. significantly reduced the above-ground biomass of *E. angustifolium* seedlings ($P < 0.05$). Similarly, disease rating was greater for plants subjected to this formulation (Table 1). Above-ground biomass was decreased by almost 30% compared

Table 1. Effect of a formulated *Alternaria* isolate on above-ground biomass and disease severity of *Epilobium angustifolium*.

Treatment	Plant above-ground biomass (g)	Disease rating[z]
Water	1.4 a[y]	0
Formulation without fungus	1.3 a	1.4
Formulation with fungus[x]	0.9 b	2.8

[x] Fungus applied at $5x10^6$ conidia m^{-2}.

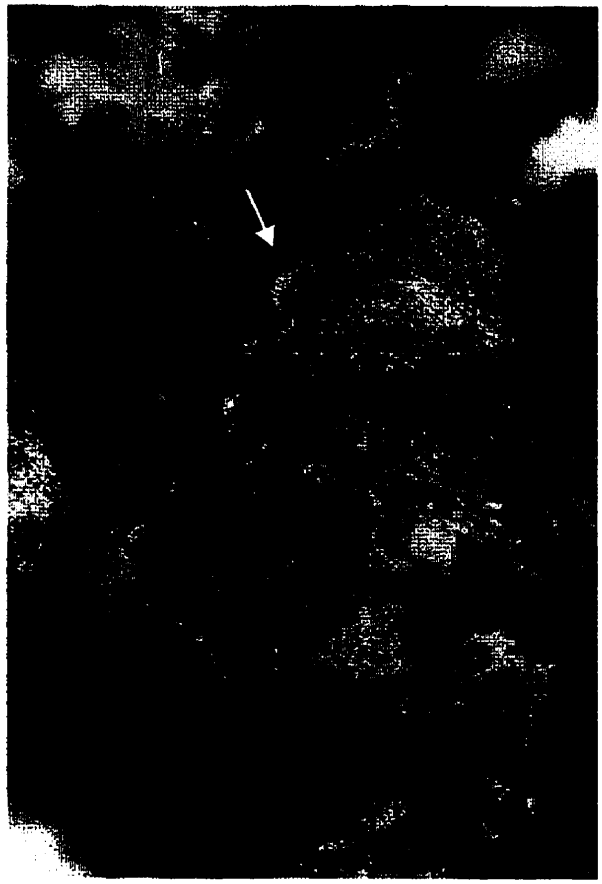
[y] Means having the same letters in a column are not significantly different according to the Student-Newman-Keuls multiple range test (SNK) at $P = 0.05$.

[z] Disease severity based on a scale from 0 to 4 where 0 = no visible symptom and 4 = > 75% necrosis.

Figure 1. Effect of a formulated *Alternaria* isolate on 5-wk-old *Epilobium angustifolium* seedlings: (**a**) one week following inoculation, formulation control on the left; (**b**) and (**c**) were taken 12 h and 1 week following inoculation respectively. Inoculated plants were provided a 12-h dew in the dark at 21 C and the formulation was applied with 5×10^6 conidia m^{-2}.


a
b
c

Figure 2. Mycelial growth observed on *Epilobium angustifolium* plants subjected to the formulated *Alternaria* isolate. Picture was taken 12 h following inoculation. Plants were provided a 12-h dew in the dark at 21 C.



with the corresponding measurements of both control treatments. Despite extensive foliar damage, the formulation of the *Alternaria* isolate was not effective in inducing plant mortality under the experimental conditions, and regrowth occurred after two weeks. Disease symptoms slowly expanded on seedlings subjected to the oil-based ground substrate formulation. Typical symptoms, characterized by tan lesions and magenta discolouration surrounding dark colonized substrate particles, appeared nearly 48 h following inoculation, and gradually resulted mostly in large and irregular foliar lesions after one week (Fig. 1). Seedlings subjected to the formulated control treatment were slightly damaged with reddish and yellow spots observed. However, these symptoms did not have any significant impact on the above-ground biomass of *E. angustifolium* seedlings. Aerial mycelium was observed emerging from the inoculated ground substrate when plants were removed from the dew chamber (Fig. 2), but disappeared as soon as the plants were returned to the growth cabinet.

6.5. Discussion

The ground colonized solid substrate of the *Alternaria* isolate formulated in an emulsified oil suspension constitutes a major improvement in the biocontrol project of *E. angustifolium*. The inoculum density used to achieve control of *E. angustifolium* seedlings was about 500 times lower than that of *Colletotrichum dematium* f.sp. *epilobii*, another bioherbicide candidate for *E. angustifolium* (Léger *et al.*, 1995). The bioherbicidal efficacy of such a low inoculum rate was presumably a consequence of the action of the oil, which by modifying the leaf surface integrity (Hull *et al.*, 1982), could have facilitated fungal

penetration and establishment (Amsellem *et al.*, 1991). Amsellem *et al.* (1990) found similar reductions in inoculum threshold with an oil-based formulation. It can be presumed that the reported *E. angustifolium* biomass reduction would be higher if healthy areas could have been segregated from damaged parts.

This formulation of the *Alternaria* isolate possesses other substantial advantages. Since the oil emulsion may have improved the wetting characteristics on the leaf surface, protected the conidia, and provided some water retention (Auld, 1993), this formulation was not dependent on a long dew period for disease initiation. Results demonstrated that its post-emergence application induced both severe damage and biomass loss to *E. angustifolium* seedlings when provided with only a 12-h dew. In addition, the viscous nature of the formulation enhanced adherence to the plant surface thereby reducing inoculum loss often observed with the application of unformulated conidial suspensions. Therefore, this formulation should improve the efficacy and reliability of *Alternaria* sp. to control *E. angustifolium* plants under field conditions.

This new formulation utilizing colonized ground solid substrate was equally applicable for *C. dematium* f.sp. *epilobii* (data not presented). This suggests that this formulation could be utilized with other microbial biological control agents.

The ground colonized solid substrate of the *Alternaria* isolate formulated in an emulsified oil suspension may be useful for fungal inoculum storage, thereby ensuring its stability over a longer period of time. The potential of using colonized seed substrate as a delivery system for other bioherbicide candidates is being investigated at the Biopesticides Research Laboratory of McGill University, and studies indicate that long term viability and

effective weed control can be achieved with pre-emergence application (Susanne Vogelgsang, pers. comm.). However, the present study constitutes the first report of using such colonized substrate material as a post-emergence treatment.

Agricultural materials, such as seeds, constitute a pool of solid substrates that can be used for bioherbicide propagule production (Miller and Churchill, 1986). Available nutrients in the colonized substrate, which were part of the sprayed formulation, may account for the presence of fungal mycelium that was observed on treated leaf surface. Nutrient depletion can enhance germination, and possibly, aggressiveness of fungal plant pathogens (Fernando *et al.*, 1996). Therefore, it can be hypothesized that this formulation provides supplemental nutrients that may favour vegetative growth rather than conidia formation, germination, and penetration, and that its efficacy would have been greater if the fungus had been cultured for a longer time.

Both the short dew duration and inoculum rate requirements and the presumably greater stability of this fungal pathogen formulation demonstrate a great potential. However, the precise role of the *Alternaria* isolate formulation for the biocontrol of *E. angustifolium* awaits further investigation. The type of agricultural material, particle size, oils, emulsifiers, and environmental factors are some of the parameters that should be examined. In addition, where the lack of control of mature plants was pointed out as an important issue in dealing with the control of *E. angustifolium* using *C. dematium* f.sp. *epilobii* (Chapter 3), the bioherbicidal activity of this formulation should be investigated in conjunction with plant age.

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Chapter 7

General discussion and conclusion

Forest managers are being required to reevaluate conventional vegetation management practices and the biocontrol strategy, specifically the bioherbicide approach, has a viable niche. The research presented in this thesis was conducted to evaluate the potential of a *Colletotrichum dematium* isolate from *Epilobium angustifolium* to control this major forest weed species.

The isolate from *E. angustifolium* possesses sufficient distinct characteristics to support its taxonomic description as a *forma specialis*. Based on a comparative study with other *C. dematium* isolates that included conidial, appressorial, cultural, temperature response, and virulence to *E. angustifolium*, it is proposed that the isolate of *C. dematium* from *E. angustifolium* be designated as *Colletotrichum dematium* f.sp. *epilobii*. This designation is further supported by the absence of previous records of natural occurrence of *C. dematium* on *E. angustifolium*.

Under controlled environmental conditions, satisfactory level of control was achieved when *E. angustifolium* seedlings were inoculated with $1x10^9$ conidia m^{-2} and subjected to a 24 h of artificial dew at 24 C. The exacting environmental requirements of *C. dematium* f.sp. *epilobii* constitute a major obstacle to its widespread use as a control strategy since extended periods of leaf wetness are rarely met under natural conditions.

The formulation of *C. dematium* f.sp. *epilobii* inoculum as an oil-based emulsion was, however, shown to be effective in inducing severe damage under conditions of reduced dew durations. However, the lack of control of mature *E. angustifolium* plants is most likely to be a limitation, and indicates that the timing of application should be carried out in the early part of the growing season. Studies conducted on both inoculum pH and buffer type provided indications of their importance for disease expression of *C. dematium* f.sp. *epilobii*. The enhancement in the level of control of *E. angustifolium* seedlings when the conidial suspension of *C. dematium* f.sp. *epilobii* was buffered at an acidic pH (3.0) and the inhibitory effect when the inoculum was adjusted to higher pH levels (> 4.0) using the citrate-phosphate buffer should be considered in further investigations on the formulation of *C. dematium* f.sp. *epilobii*.

Host range tests suggest that *C. dematium* f.sp. *epilobii* is apparently limited to the Onagraceae family. This supports the findings of Winder and Watson (1994), and reinforces the conclusion that the isolate from *E. angustifolium* is a *forma specialis*. In addition to *E. angustifolium*, only *Camissonia bistorta* and *Clarkia unguiculata* were susceptible to the fungus under optimum controlled conditions. The fact that one collection of *E. angustifolium*, from Oka, Québec, was not susceptible to *C. dematium* f.sp. *epilobii* illustrates the potential variability of control of *E. angustifolium* under natural conditions. Since tree species of economic importance in Canada inoculated with the most promising formulations of *C. dematium* f.sp. *epilobii* were immune under field conditions, with the exception yellow birch (*Betula alleghaniensis*), this fungus could be used selectively in reforestation areas.

Under field conditions, the successful growth suppression of *E. angustifolium* seedlings with the oil-based formulation of *C. dematium* f.sp *epilobii* in a tank-mix combination with a low rate of glyphosate provides another strategy for future development. The ability of glyphosate to translocate to underground propagules would restrict, in contrast to the fungus, the regenerative ability of this perennial weed species. However, the observed lack of control of mature plants reinforces the need to focus future studies on control of *E. angustifolium* by *C. dematium* f.sp *epilobii* earlier in the growing season.

Under controlled environmental conditions, post-emergence application of an oil emulsion containing the ground colonized substrate of an *Alternaria* sp., another bioherbicide candidate for *E. angustifolium*, was found to be effective in inducing severe damage to *E. angustifolium* seedlings at a low dew duration (12 h) and inoculum density ($5x10^6$ conidia m^{-2}). However, the potential of the formulation of this *Alternaria* isolate for the biocontrol of *E. angustifolium* awaits further investigation.

C. dematium f.sp *epilobii* has a potential to be further developed as a bioherbicide for *E. angustifolium*. When properly formulated and strategically applied, this fungus provides some control of *E. angustifolium* growth. Therefore, its potential should be considered within existing vegetation management practices, particularly with the use of chemical methods.

Suggestions for future research

1. Integrate *C. dematium* f.sp. *epilobii* with low rates of chemical herbicides.
2. Evaluate more fully the potential of the formulation utilizing colonized ground solid substrate, both with the *Alternaria* isolate and *C. dematium* f.sp. *epilobii*.
3. Improve the control of mature *E. angustifolium* plants with a suitable formulation.
4. Test different timing of application in the field.

APPENDIX

Experiments not discussed in the text

Additional experiments were conducted during the course of the studies. Results are presented in the appendix. The purpose of these studies was to improve experimental procedures. The first experiment (Tables 1 and 2) dealt with the effect of air moisture, seed sterilization, and type of soil on the emergence of *E. angustifolium* seedlings. The second experiment (Table 3) was conducted with the objective to evaluate solid agricultural products for conidial production of *C. dematium*.

Table 1. Effect of three seed treatments on emergence of *Epilobium angustifolium* in three covered substrates.[w]

Day	Treatment[x] / substrate[y]								
	0 / A	5 / A	10 / A	0 / B	5 / B	10 / B	0 / C	5 / C	10 / C
1	0[z]	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	4
4	0	0	0	0	0	0	0	0	4
5	4	20	0	12	12	20	0	20	24
6	12	32	0	24	36	40	0	44	44
7	12	48	8	24	56	60	4	60	64
8	12	56	16	28	72	76	4	84	88
9	40	16	28	36	72	80	12	84	92
10	40	32	36	44	100	84	16	100	92
11	44	44	52	68	100	84	24	100	92
12	44	52	52	68	100	100	36	100	92
13	52	56	52	68	100	100	40	100	92
14	60	60	52	68	100	100	40	100	92
15	72	64	36	68	100	100	40	100	92
16	76	68	36	68	100	100	40	100	92
17	84	68	44	72	100	100	40	100	92
18	84	68	52	72	100	100	40	100	92
19	84	68	56	72	100	100	40	100	92
20	84	68	56	72	100	100	40	100	92
21	84	68	56	72	100	100	40	100	92

[w] Flats covered to maintain high relative humidity.

[x] 0 = unsterilized.
5 = 5 min seed sterilization (5% Javex solution).
10 = 10 min seed sterilization (5% Javex solution).

[y] A = Promix.
B = sand / Promix / loam (1:1:1).
C = sand / Promix / loam (1.25:0.5:1.25).

[z] Results expressed as percent emergence.

Table 2. Effect of three seed treatments on emergence of *Epilobium angustifolium* in three uncovered substrates.

Day	Treatment[x] / substrate[y]								
	0 / A	5 / A	10 / A	0 / B	5 / B	10 / B	0 / C	5 / C	10 / C
1	0[z]	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	4	0	0	0
6	12	0	4	0	0	20	16	8	0
7	24	4	16	0	0	32	32	16	0
8	40	16	28	0	0	52	36	16	4
9	56	28	28	0	0	60	48	20	8
10	56	40	36	4	4	60	52	48	20
11	56	52	36	12	12	60	52	48	20
12	56	60	36	16	12	60	52	48	24
13	56	68	36	20	12	60	52	48	24
14	56	68	36	24	20	60	48	52	28
15	60	68	36	24	20	60	40	60	28
16	68	68	36	24	20	64	40	60	28
17	76	68	40	24	20	64	40	60	28
18	76	72	44	24	20	68	40	60	28
19	80	76	48	24	20	68	40	44	28
20	80	76	48	24	20	68	40	44	28
21	80	76	48	24	20	68	40	44	28

[x] 0 = unsterilized.
5 = 5 min seed sterilization (5% Javex solution).
10 = 10 min seed sterilization (5% Javex solution).

[y] A = Promix.
B = sand / Promix / loam (1:1:1).
C = sand / Promix / loam (1.25:0.5:1.25).

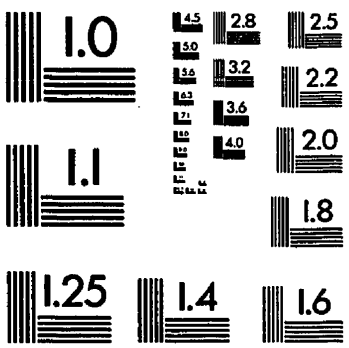
[z] Results expressed as percent emergence.

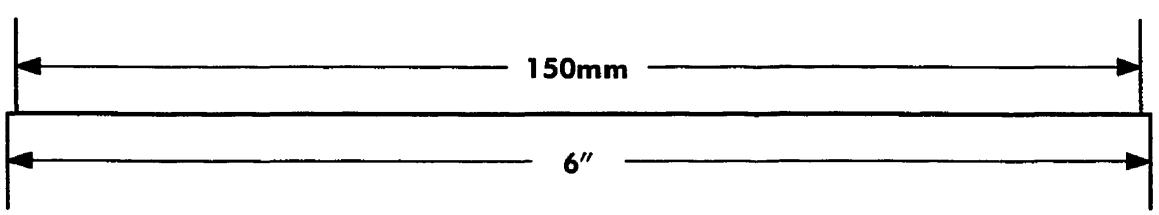
Table 3. *Colletotrichum dematium* f.sp. *epilobii* conidia production (spore g^{-1}) on agricultural based solid substrates.[z]

Solid substrate	Incubation period (days)			
	7	14	21	28
Black eyed bean	1.94×10^4	2.22×10^4	3.06×10^4	9.72×10^4
Chickpea	3.89×10^4	2.22×10^4	6.86×10^6	7.89×10^5
Rice grain	2.06×10^6	1.58×10^8	7.89×10^7	8.45×10^7
Millet	1.09×10^7	$\mathbf{1.62 \times 10^8}$	3.12×10^8	3.74×10^7
Mungbean	0	1.39×10^4	3.89×10^4	1.11×10^4
Pearl barley	8.33×10^5	6.87×10^7	9.17×10^7	4.10×10^8
Pot barley	6.67×10^6	1.76×10^8	1.26×10^6	3.90×10^6
Quinoa	2.45×10^5	1.46×10^7	1.50×10^8	8.86×10^8
Red lentil	1.39×10^4	2.78×10^4	3.89×10^4	2.78×10^4
Wheat	2.46×10^5	8.47×10^6	5.01×10^7	1.34×10^8
Soybean	5.56×10^3	2.78×10^3	8.89×10^4	2.94×10^5
Yellow pea	0	1.53×10^5	2.58×10^5	1.11×10^5

[z] Experimental procedures follow those described in section 3.3.1 of chapter 3.

TEST TARGET (QA-3)





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