

Dissertationes Forestales 93

The efficacy and potential risks of controlling sprouting
in Finnish birches (*Betula* spp.) with the fungal
decomposer *Chondrostereum purpureum*

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Academic dissertation

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ABSTRACT

Sprouting of fast-growing broad-leaved trees causes problems in young coniferous stands, under power transmission lines and along roads and railways. Public opinion and the Finnish Forest Certification System oppose the use of chemical herbicides to control sprouting, which means that most areas with problems rely on mechanical cutting. However, cutting is a poor control method for many broad-leaved species because the removal of leaders can stimulate the sprouting of side branches and cut stumps quickly re-sprout. In order to be effective, cutting must be carried out frequently but each cut increases the costs, making this control method increasingly difficult and expensive once begun. As such, alternative methods for sprout control that are both effective and environmentally sound represent a continuing challenge to managers and research biologists.

Using biological control agents to prevent sprouting has been given serious consideration recently. Dutch and Canadian researchers have demonstrated the potential of the white-rot fungus *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar as a control agent of stump sprouting in many hardwoods. These findings have focused the attention of the Finnish forestry community on the utilization of *C. purpureum* for biocontrol purposes.

Primarily, this study sought to determine the efficacy of native *C. purpureum* as an inhibitor of birch stump sprouting in Finland and to clarify its mode of action. Additionally, genotypic variation in Finnish *C. purpureum* was examined and the environmental risks posed by a biocontrol program using this fungus were assessed.

Experimental results of the study demonstrated that *C. purpureum* clearly affects the sprouting of birch: both the frequency of living stumps and the number of living sprouts per stump were effectively reduced by the treatment. However, the treatment had no effect on the maximum height of new sprouts. There were clear differences among fungal isolates in preventing sprouting and those that possessed high oxidative activities in the laboratory inhibited sprouting most efficiently in the field. The most effective treatment time during the growing season was in early and mid summer (May–July).

Genetic diversity in Nordic and Baltic populations of *C. purpureum* was found to be high at the regional scale but locally homogeneous. This natural distribution of diversity means that using local genotypes in biocontrol programs would effectively prevent the introduction of novel genes or genotypes. While a biocontrol program using local strains of *C. purpureum* would be environmentally neutral, pruned birches that are close to the treatment site would have a high susceptibility to infect by the fungus during the early spring.

Keywords: White-rot fungus, biological control, vegetation management, biocontrol agent, herbicide alternative.

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Vantaa, May 2009

Henna Vartiamäki

LIST OF ORIGINAL ARTICLES

The thesis is based on the following articles, which are referred to by their Roman numerals (I–IV) in the text.

- I** Vartiamäki, H., Uotila, A., Vasaitis, R. and Hantula, J. 2008. Genetic diversity in Nordic and Baltic populations of *Chondrostereum purpureum*: a potential herbicide biocontrol agent. *Forest Pathology* 38: 381-393.
- II** Vartiamäki, H., Maijala, P., Uotila, A. and Hantula, J. 2008. Characterization of growth and enzyme production of *Chondrostereum purpureum* isolates and correlation of these characteristics with their capability to prevent sprouting of birch in field. *Biological Control* 47: 46-54.
- III** Vartiamäki, H., Hantula, J. and Uotila, A. 2009. Effect of application time on the efficacy of *Chondrostereum purpureum* treatment against the sprouting of birch in Finland. *Canadian Journal of Forest Research* 39: 731-739.
- IV** Vartiamäki, H., Hantula, J. and Uotila, A. Susceptibility of Silver birch pruning wounds to infection by white-rot fungus (*Chondrostereum purpureum*), a potential bioherbicide. *Silva Fennica* (in press).

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AUTHOR'S CONTRIBUTION

- I** Henna Vartiamäki planned the work with Jarkko Hantula. Vartiamäki collected the samples, except those from Lithuania provided by Rimvydas Vasaitis. Vartiamäki did most of the laboratory work and the data were analysed by Hantula and Vartiamäki. Vartiamäki had the main responsibility for the writing process. The coauthors participated in the writing process and discussion of the results.
- II** Henna Vartiamäki planned and conducted experiments in the laboratory together with Pekka Maijala. Vartiamäki designed the field trials together with Antti Uotila. Vartiamäki and Uotila established the field trials. Vartiamäki was responsible for collecting the field data, its analysis and preparing the first draft of the manuscript. The coauthors participated in the writing process and discussion of the results.
- III, IV** Henna Vartiamäki designed and established the field trials with Antti Uotila. Vartiamäki was responsible for collecting the field data, its analysis and preparing the first draft of the manuscript. The coauthors participated in the writing process and discussion of the results.

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1 INTRODUCTION

1.1 The problem of sprouting

Sprouting of fast-growing broad-leaved trees causes problems in many areas, such as in regeneration areas and young seedling stands. In those areas broad-leaved trees compete for light, water, nutrients and space with more valuable coniferous species. Moreover, rapid growth of sprouts also causes problems under power transmission lines and along roadsides and railways.

Understandably, sprouting of several broad-leaved species causes problems in Finland, particularly silver birch (*Betula pendula* Roth), downy birch (*Betula pubescens* Ehrh.), aspen (*Populus tremula* L.), rowan (*Sorbus aucuparia* L.) and willows (*Salix* L. spp.). Since 80% of broad-leaved trees in Finland are downy and silver birches (Peltola 2007), these species account for most problems. Vegetative reproduction of all broad-leaved species occurs either via stump or root sprouts but their sprouting ability varies greatly (Lust and Mohammady 1973). Birches regenerate by producing numerous stump sprouts from dormant basal buds (Kauppi et al. 1991) located mostly underground (Kauppi et al. 1987, Johansson 1992). Cutting season, size and age of the trees, stump height and site quality can significantly affect the early development of birch sprouts (Etholén 1974, Ferm and Issakainen 1981, Johansson 1992).

Sprouting ability of downy birch is slightly greater than silver birch (Johansson 1992, Johansson 2008). In Johansson's (2008) study, 90% of downy birch stumps and 82% of silver birch stumps resprouted 1 year after being cut. The number of living stumps decreased in years following cuts and nine years after cutting only 61% of downy birch stumps and 55% of silver birch stumps were resprouting.

1.2 Preventing sprouting

1.2.1 Different methods for preventing sprouting

Sprouting can be prevented using four different kinds of methods: 1) mechanical cutting, 2) herbicides or bioherbicides, 3) up-rooting, or 4) topping (Tham 1983). In Finland, mechanical cutting is the most commonly used sprout control method. Prior to the recognition of their harmful impact on the environment in the 1980s, chemical herbicides such as glyphosate were widely used to control sprouting. Since then, environmentally-safe alternatives to herbicides (biocontrol agents or bioherbicides) have been under development. The other methods of up-rooting and topping are not commonly used sprout control programs, perhaps because they are quite difficult to carry out in large scale.

1.2.2 Mechanical cutting

Mechanical cutting is one of the oldest vegetation management tools. However, mechanical cutting is rather ineffective in sprouting control since broad-leaved trees resprout quickly after cutting and the treatment itself can stimulate vigorous regrowth. In order to be effective, mechanical cutting must be carried out periodically and each repetition increases the cost of subsequent treatments. Along roadsides and under power lines, cutting must be repeated

every few years. In forestry situations and under power transmission lines, mechanical cutting is carried out manually with clearing saws, whereas clearance machines are commonly used along roadsides. Areas cut with machines have taller and more sprouts than areas cut with clearing saws (Johansson 1991).

In conifer stands, mechanical cutting can be done only once if the timing is well chosen and effectively carried out. The number of sprouts and their height growth are dependent on the time when the cutting is carried out. This is believed to be the result of a seasonal fluctuation in carbohydrate reserves (Aldous 1929, Johansson 1993). For birch, cutting during the growth period diminishes the number of new sprouts and minimizes their height compared with cuts made at other times (Stoekler 1947, Johansson 1992).

1.2.3 Chemical herbicides

Chemical herbicides are designed to be highly toxic to a target species but otherwise neutral to the rest of the biological community. Ideally, they should only affect the area where they are applied and the effect should last as short a time is necessary to kill or control the target species. In practice, however, herbicide often enters the atmosphere, soil, aquatic environment and even other organisms. In addition, chemicals can be spilled due to careless storage or handling of the substance or its waste products. As such, chemical herbicides can have harmful side effects as a result of incidental and accidental release into the environment.

In forestry, chemical herbicides are traditionally used for controlling grass and tree sprouts, especially in afforested locations previously used for agriculture (Jylhä and Hytönen 2006). Depending on scale, herbicides can be applied manually, by wheeled machinery or by airplane. The Finnish Forest Certification System (FFCS) states that chemicals should be avoided in modern silviculture (Metsäsertifiointin uudistetut... 2005) but tolerates their use in places where sprouting is exceptionally vigorous. Thus, the current application of chemicals in Finland is restricted.

1.2.4 Biological vegetation control

Since mechanical cutting is considered an ineffective method for preventing sprouting and the use of chemical herbicides is tightly restricted, the development of alternative and more efficient methods for sprout control is a continuing challenge. A promising direction concerns the use of biological agents in tandem with mechanical cutting.

Biological control methods rely on a natural agent to reduce or eliminate an unwanted organism (Van Driesche and Bellows 1996). In other words, one organism is employed to suppress another. The idea of using biological control in vegetation management is not a new concept, and the principle of biological control has been developed over the past 100 years mainly by entomologists using natural enemies of pest insects (Waage 1997).

Biological control of vegetation traditionally relies upon importing the natural enemies of introduced weeds (classical biological control strategy). Although the classical strategy is widely used in agriculture, forest managers have preferred to give a selective advantage to economically important species because they are in competition with native rather than exotic species.

A nonclassical biological control method (inundative biocontrol strategy) has been used successfully to control native plants in agriculture (Charudattan 2001). The inundative biocontrol strategy is based on the use of indigenous fungal or bacterial pathogens (mycoherbicides, bioherbicides) that are formulated and mass-produced. They are applied to

target vegetation in a manner similar to chemical herbicides and they repress or eliminate the growth of the target species (Templeton et al. 1979, Templeton and Greaves 1984).

For agriculture control programs, plant pathogens developed for commercial use (mycoherbicides) include the pathogenic fungus *Phytophthora palmivora* (DeVine®) for the control of strangler vine (*Morrenia odorata*) in citrus groves and *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (COLLEGO®) for managing northern jointvetch (*Aeschynomene virginica*) in rice (*Oryza sativa*) and soybean (*Glycine max*) fields (Templeton and Heiny 1990).

For forestry control programs, mycoherbicides represent a feasible and environmentally acceptable alternative to chemical herbicides. Plant pathogens that have been tested for use in forestry situations include the pathogenic fungus *Colletotrichum dematium* f.sp. *epilobii* for the control of fireweed (*Epilobium angustifolium*) (Léger et al. 2001) and *Valdensinia heterodoxa* for the control of salal (*Gaultheria shallon* Pursh.) (Vogelgsang and Shamoun 2002).

In sprout control of broad-leaved trees, one potential biocontrol agent is the basidiomycete plant pathogen *Chondrostereum purpureum* (Pers. ex Fr.) Pouzar (Syn. *Stereum purpureum* Pers., *Thelephora purpurea* (Pers.) Pers., *Auricularia persistens* Sowerby, *Stereum vorticosum* (Fr.) Fr.). Freshly cut stumps receive an application of mycelia that suppresses sprout regrowth (Wall 1990). Researchers in the Netherlands and Canada have demonstrated the potential of *C. purpureum* for the control of stump sprouting in many broad-leaved tree species (Wall 1986, 1990, Scheepens and Hoogerbrugge 1989, De Jong et al. 1990a, Gosselin 1996, Dumas et al. 1997, Shamoun and Hintz 1998, Harper et al. 1999, Pitt et al. 1999, Hintz 2007). Control programs based on this information have increased the time interval between required cuts at sites treated with *C. purpureum*. The use of *C. purpureum* to control sprouting in Finland is an attractive proposition. However, there is no information on the utility of North European *C. purpureum* as a biocontrol agent of the native tree species and under local climatic conditions.

1.3 *Chondrostereum purpureum*

1.3.1 Biology of *C. purpureum*

Chondrostereum purpureum is a lignicolous, basidiomycete fungus, distributed in temperate regions of the Northern and Southern Hemispheres. It is a facultative saprophyte with broad-spectrum pathogenicity towards many broad-leaved tree species (Rayner and Boddy 1986). *Chondrostereum purpureum* is also known as a causative agent of silver-leaf disease in apple (*Malus* Mill.), pear (*Pyrus* L.), plum (*Prunus* L.), cherry (*Prunus* L.) and other fruit trees (Brooks and Moore 1926). Symptoms arise when mycelia occlude xylem vessels and block the transfer of sap to the leaves (Setliff and Wade 1973, Bishop 1979). It can also invade coniferous wood as a saprophyte but it is not known to cause disease in healthy conifers (Etheridge and Morin 1963). In Finland, it is most commonly found on fresh stumps of birch (*Betula* L.) (45% of cases), aspen (*Populus* L.) (17%) and alder (*Alnus* Mill.) (11%) (Kauppila and Niemelä 1986), and its apparent preference for Betulaceae was also found in North America (45%) (Setliff 2002) and Norway (36%) (Ryvander 1971).

Chondrostereum purpureum is a primary invader of freshly wounded tissue such as those created by pruning, felling, wind damage and frost cracks. Symptoms of infection include sapwood discoloration, decay and sometimes death (Rayner 1977, Wall 1986, 1991). On

stumps, *C. purpureum* functions as a decay promoter that is quickly replaced by other more competitive fungi (Rayner 1978, 1979, Gosselin 1996).

Chondrostereum purpureum disseminates through the production of numerous basidiospores from fertile fruiting bodies (basidiocarps or sporophores), which it produces on recently dead wood (Dye 1974). The fruiting body is normally pileate or resupinate and white and tomentose above. The hymenium is smooth and dark violet, purplish or brown-violet when fresh and wet; after drying it is paler. In vertical section, the white tomentum of the pileus is separated from the lower layers by a dark line visible to the naked eye. Under a lens, the fruiting body is composed of 5 or 6 different layers (Eriksson and Ryvander 1976).

Under suitable conditions, *C. purpureum* usually fructificates two consecutive years after infection (Wall 1997) and spores are released for several months (Dye 1974). Rainfall and relative humidity are the key factors in sporophore formation and basidiospore release (Dye 1974, Spiers 1985, Spiers and Hopcroft 1988b). Sporophores produce spores only after immersion in free water or if grown on a substrate with greater than 75% moisture content (Spiers 1985). The airborne basidiospores are short-lived (<5h), probably due to sensitivity to desiccation and ultraviolet (UV) radiation (Grosclaude 1969). However, if they reach a moist wounded surface they may germinate and the resulting hyphae can penetrate and ramify through the plant tissues inter- and intracellularly (Spiers and Hopcroft 1988a).

Chondrostereum purpureum is heterothallic and has a tetrapolar mating system (Boidin 1971, Wall et al. 1996). This type of reproduction promotes outbreeding because successful conjugation occurs between two genetically distinct but compatible mycelia. Sexual compatibility studies between single-spore isolates suggest the presence of multiple alleles at two mating-type loci (A and B). Vegetative incompatibility barriers have not been observed; hence a fertile heterokaryotic mycelium can be formed in all sexually compatible matings. In homokaryotic isolates from widely separated regions, mating should be 100% successful because the multiallelic nature of the A and B factors ensure compatibility between isolates (Boidin 1986). Clamp connections are characteristic of the heterokaryotic mycelium (Eriksson and Ryvander 1976).

Plant pathogenic microorganisms must repeatedly encounter and penetrate the cell walls of their hosts. Most but not all parasites can produce extracellular degrading enzymes capable of cleaving the major glycosidic linkages in plant cell walls. In *C. purpureum*, the potential virulence factors or determinants for their efficiency as sprout-controlling organisms are poorly characterized. However, the purified pectinase of *C. purpureum* induced silver-leaf disease symptoms in apple seedlings (Miyairi et al. 1985). Production of phytotoxins is also associated with foliar symptoms of silver-leaf disease (Bishop 1979).

1.3.2 *Chondrostereum purpureum* as a biocontrol agent

Chondrostereum purpureum is well suited for biological sprout control because it shows broad-spectrum pathogenicity towards many hardwood species and, because it is globally distributed in temperate regions of the Northern and Southern Hemispheres, concerns over its introduction as an alien are largely moot. Additionally, since it is a fast growing fungus its mycelium can be produced efficiently *in vitro*.

In the Netherlands, *C. purpureum* has been used as a vegetation management tool since the 1980s (Scheepens and Hoogerbrugge 1989) and is currently in trials as a mycoherbicide product under the brand name BioChon. However, the product is not yet registered for commercial use. BioChon generally kills about 95% of the stumps of rum cherry (*Prunus*

serotina Ehrh.) two years after treatment and appears to be as effective on hybrid black poplar (*Populus X canadensis* Moench) (De Jong 2000).

In Canada, *C. purpureum* has also been investigated as a mycoherbicide since the 1980s (e.g., Wall, 1986, 1990, 1991). Myco-Tech™ Paste (Myco-Forestis Corp., L'Assomption, Quebec), a commercial stump-resprouting inhibitor of broad-leaved trees, has been registered in Canada since 2002 (Pest Management Regulatory Agency [PMRA] 2002). Myco-Tech™ Paste is a formulated product containing viable mycelium of *C. purpureum* but not spores. Artificial inoculation has proved to be more successful with mycelium than with spores (Spiers and Hopcroft 1988a). The gel is applied as a thin layer over the exposed surface of freshly cut broad-leaved tree stumps within 30 minutes of cutting. A second Canadian product, Chontrol™ Paste (MycoLogic Inc., University of Victoria, Victoria, British Columbia) has been registered for use since 2007 (Pest Management Regulatory Agency [PMRA] 2007). The efficacy of these two *C. purpureum* products has been evaluated for the control of red maple (*Acer rubrum* L.), sugar maple (*A. saccharum* Marsh.), Oregon maple (*A. macrophyllum* Pursh), red alder (*Alnus rubra* Bong.), yellow birch (*Betula alleghaniensis* Britton), paper birch (*B. papyrifera* Marsh.), beech (*Fagus grandifolia* Ehrh.), American aspen (*Populus tremuloides* Michx.), bigtooth aspen (*P. grandidentata* Michx.) and pin cherry (*Prunus pensylvanica* L. f.) (Dumas et al. 1997, Jobidon 1998, Harper et al. 1999, Pitt et al. 1999, Wall 1986, 1990, 1991). The results of the field trials showed that *C. purpureum* can have an efficacy comparable to that of chemical herbicides. On red alder, for example, 92% of stumps inoculated with *C. purpureum* died in the first year and 100% were dead by the second year (Becker et al. 2005).

It is important to perform the treatment with *C. purpureum* immediately after stumps are cut. Freshly cut stumps of healthy trees provide a microbiological vacuum and are particularly vulnerable to fungal infection (Rayner 1977). However, trees have developed defense mechanisms to protect themselves against infection as well as desiccation. Wounding stimulates the parenchyma cells to form a barrier between living tissues and the environment. The suberization process starts in the radial and axial parenchyma cells near the wound and fibril plugs in the vessels form a protective layer. Eventually, the entire lamina of the wound becomes occluded and fungal invasion becomes more difficult (Schmitt and Liese 1990, 1991, 1993). Brooks and Moore (1926) showed that fresh wounds on pear trees were highly susceptible to infection by *C. purpureum* whereas it was very difficult to infect month-old wounds and impossible to infect 3-month-old wounds.

1.3.3 Environmental risks of using *C. purpureum* as a biocontrol agent

The general use of Canadian *C. purpureum* products elsewhere in the World, e.g., in Finland, is questionable because of uncertainties about their action in a different environment and concerns over the introduction of new genetic material into the local population. New genetic material could introduce rare and virulent alleles to the local population of the pathogen, resulting in a general increase in its infectivity and pathogenicity (Burdon and Silk 1997, Hintz et al. 2001).

Indeed, such concerns are supported by real-world cases. In 2002, an exotic population of the North American *Heterobasidion annosum* intersterility group P (ISG P) was detected in a single stone pine (*Pinus pinea* L.) stand in Italy (Gonthier et al. 2004). A few years later, this distribution had expanded to an area 100-km-long and 27-km-wide range of expansion (Gonthier et al. 2007). The North American *H. annosum* ISG P aggressively forms large disease gaps in the pine forests of Italy and its frequent occurrence suggests that it is better adapted to the ecological conditions in this region than the native European P group (D'Amico

et al. 2007, Gonthier et al. 2007). This example encourages the use of native strains of *C. purpureum* for local biological control programs.

Since *C. purpureum* can survive as a saprophyte on wood, it can be assumed that there is little selection pressure towards greater virulence or host specialization. The fungus is distributed in temperate regions of the Northern and Southern Hemispheres and, therefore, it can be assumed that its dispersal capacity is high. The population structure of *C. purpureum* has been studied from regional to continental scales using diverse methods to differentiate the subgroups such as mating interactions (Rayner and Boddy 1986), sodium dodecyl sulphate (SDS)-protein profiles (Ekramoddoullah et al. 1993), isozyme analyses (Shamoun and Wall 1996), ribosomal DNA and mitochondrial DNA (mtDNA) profiles (Ramsfield et al. 1996, 1999), random amplification of polymorphic DNA (RAPD) (Gosselin et al. 1996) and a combination of morphology, pathogenicity and RAPD (Spiers et al. 2000). These studies concluded that the *C. purpureum* population is panmictic and has no geographic or host specialization in the area studied. Therefore, in countries where *C. purpureum* is native, there appears to be little danger of introducing new genes to local populations through the use of bioherbicide products from other geographic regions within the same continent.

Chondrostereum purpureum is a good candidate for biocontrol purposes because it has a wide host range. However, incidental infection of nontarget species due to its application as a biocontrol agent would be undesirable. Studies have indicated a low risk of incidental infection due to application of *C. purpureum* mycelium (Becker et al. 1999a, b) but infection via spores originating from the treated stumps has been a matter for concern (De Jong 1992, De Jong et al. 1990a, b, Gosselin et al. 1999). On treated stumps, the fungus usually produces fruiting bodies up to 2 years (Wall 1997). While basidiospores can infect freshly wounded broad-leaved trees as well as fruit or ornamental trees, De Jong et al. (1990b) concluded that the risk of infection more than 500 m from the target area was very low.

Nontarget infection risk could be minimized if spore dispersal was reduced. De Jong (2000) examined whether the spore dispersal could be reduced by preventing fructification of *C. purpureum* through the addition of saprophytic wood-invading fungi (*Stereum hirsutum* (Willd.) Pers. or *Nectria cinnabarina* (Tode) Fr.) to the inoculum. Unfortunately, they had no effect. Additionally, De Jong et al. (1998) studied the use of single-spore isolations for inoculations but found that also single-spore isolates produced basidiocarps with viable spores. Single spore isolates were also as pathogenic as multiple spore cultures.

2 AIMS OF THE STUDY

Researchers in the Netherlands and Canada have shown that the decay fungus *Chondrostereum purpureum* can be used to successfully control stump sprouting of many broad-leaved trees (Scheepens and Hoogerbrugge 1989, De Jong 2000, Wall 1990, Shamoun and Hintz 1998, Harper et al. 1999, Pitt et al. 1999). However, the utility of North European *C. purpureum* as a biocontrol agent has not been tested with respect to the local climate conditions and plant community.

Primarily, this study sought to determine the potential use of native *C. purpureum* as an inhibitor of birch stump sprouting in Finland (**I**, **II**, **III**) and to clarify its mode of action (**II**). Additionally, genotypic variation in Finnish *C. purpureum* was examined (**I**) and the environmental risks posed by a biocontrol program using *C. purpureum* were assessed (**I**, **IV**). Birches were chosen as the experimental tree species because about 80% of all broad-leaved trees in Finland are *Betula* (Peltola 2007).

The hypotheses tested were:

1. There is high genetic variability within *C. purpureum* in Nordic and Baltic countries (**I**).
2. *In vitro* enzyme activity and biomass production correlate positively with the efficacy of *C. purpureum* in the field (**II**).
3. Different isolates of *C. purpureum* have different potencies in preventing birch sprouting (**II**).
4. Timing of application with respect to the growing season affects the efficacy of *C. purpureum* as a biocontrol agent of birch sprouting (**III**).
5. The use of *C. purpureum* as a biocontrol agent poses environmental risks (**I**, **IV**).

3 MATERIAL AND METHODS

3.1 Fungal isolates and their maintenance (I–IV)

A total of 85 different isolates of *C. purpureum* were collected from 5 separate locations (I). Four populations originated from different parts of Finland and one population was collected from Lithuania (see I, Fig. 1). All *C. purpureum* individuals were taken from birch and from a spatially separated resource unit (stump or log). Only homokaryotic isolates were used (I). Since the absence of clamp connections is the characteristic for the homokaryotic mycelium of *C. purpureum* (Eriksson and Ryvander 1976), isolates were screened for their absence before use. Isolates were subcultured on potato dextrose agar (PDA) Petri plates covered with cellophane membrane and from which they were harvested for DNA isolation.

Twenty-one heterokaryotic isolates of *C. purpureum* were used in laboratory tests (II). Fourteen isolates were from Finland, four from Sweden and three from Lithuania. Eighteen isolates were isolated from *Betula* spp., two from *Populus* spp. and one from *Salix* spp. All fungal isolates were precultured on PDA Petri plates before laboratory testing. Eight Finnish isolates were chosen for the field trials based on their enzymatic activity and fungal biomass production in laboratory.

The heterokaryotic isolate of *C. purpureum* (P3) used in field trials (III, IV) was originally isolated from silver birch in Vantaa, southern Finland. It was isolated from a piece of basidiocarp on a PDA Petri plate, where it was also maintained and precultured before use in the field trials. Isolate P3 was selected for the field trials based on its fast growing rate on various agar Petri plates at different temperatures (data not shown) and with respect to twenty other isolates tested.

3.2 Analysis of genetic variation (I)

The DNA from the single-basidiospore isolates was isolated as described by Vainio et al. (1998) (I). For DNA fingerprinting, the random amplified microsatellites (RAMS) technique was used. Amplification products were analysed by electrophoresis in SynerGel agarose gels (Diversified Biotech, Boston, MA, USA) run in Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer and visualized using ethidium bromide under ultraviolet (UV) light. A distance matrix was constructed from the RAMS data, using RAPDistance software version 1.04 (Australian National University, Canberra, Australia) (Armstrong et al. 1994). The molecular evolutionary genetic analysis (MEGA) software (Center for Evolutionary Functional Genomics, Biodesign Institute, Tempe, AZ, USA) (Kumar et al. 2004) was used to construct a dendrogram with the unweighted pair group method with arithmetic mean (UPGMA). An analysis of molecular variance and estimate of genetic diversity within populations were carried out using the Arlequin version 3.11 (Department of Anthropology and Ecology, University of Geneva, Switzerland) (Excoffier et al. 2005). The occurrence of linkage disequilibrium between all pairs of markers was tested with Fischer's exact test using SPSS software version 16.0.

3.3 Screening of enzymatic activity (II)

The activity levels of pectinases, proteases, cellulases, lipases and lignin-degrading oxidative enzymes (laccase and manganese peroxidase) of 21 *C. purpureum* isolates were evaluated in Petri plate tests (II). Substrate plates for different enzyme activities were prepared as explained elsewhere (II). Each screening Petri plate was inoculated with a standardized agar plug containing precultured mycelium. Each combination of isolate and substrate was replicated twice, i.e., two plates. Colour change in the agar plates was evaluated after an incubation time dependent on the substrate and classified from 0 (no colour reaction) to 3 (strong colour reaction) by the same individual.

3.4 Fungal biomass production (II)

Fungal growth on wood chips was investigated using a fluorescein diacetate (FDA) assay as described elsewhere (II). FDA is a fluorogenic substrate that becomes fluorescent upon enzymatic cleavage caused by several nonspecific enzymes found in living cells and it has been used as a test of fungal viability (e.g., Söderström 1977, Schnürer and Rosswall 1982, Barak and Chet 1986, Bjurman 1993). The liberated fluorescein from each trial was detected spectrophotometrically.

3.5 Field trials (II, III, IV)

3.5.1 Study sites

All three field trial sites were located about 200 km north of Helsinki in central Finland; Juupajoki (II), Orivesi (III) and Ruovesi (IV). Sites II and III included 13-year-old spruce (*Picea* A. Dietr.) stands growing in a mixed forest with birches (*Betula pendula* and *B. pubescens*). The height of the spruces was about 2 m and that of the birches was 2–5 m (II, III). The stump diameter of birches, 20–30 cm above ground level, was about 3–4 cm (II, III). Both stands (II, III) were classified as Myrtillus type according to Cajander (1949). Site IV was a 20-year-old silver birch stand classified as an Oxalis-Myrtillus type (Cajander 1949). The average height of the birch was 9.7 m and the average breast height diameter was 10 cm (IV).

3.5.2 Inocula

The inoculum medium consisted of 12 g of potato dextrose broth, 10 g Sipernat®22S (Evonik Industries GmbH, Essen, Germany) and 500 ml of distilled water in a 1000-ml Erlenmeyer flask and was autoclaved at 121 °C for 15 min (II, III, IV). The flask was inoculated with mycelium of *C. purpureum* taken from the edge of a 7-day-old culture growing on a PDA cellophane plate and incubated in the dark at room temperature on a rotation shaker for 8–10 days. The inoculum was homogenized with an Ultra Turrax apparatus (IKA-Werke GmbH+Co. Kg, Staufen, Germany) shortly before application. The ready-made inoculum was diluted 1:10 with tap water before use. The concentrations, viabilities and purities of the inocula were confirmed before and after the field applications with the most probable number (MPN) method (Harris and Sommers 1968).

3.5.3 Experimental designs and field treatments

Study **II** was undertaken in mid-June 2006. Eight isolates were chosen for the field trial based on their performance under laboratory conditions. Forty treatment plots were established, consisting of four replicates of each treatment (eight isolates of *C. purpureum*, slash control and formulation control). The experimental design was completely randomized. Plots were circular, separated by buffer zones at least 2 m wide and variable in diameter. Plot diameter was determined by the area required to include at least 21 birch stumps each with a diameter of more than 10 mm.

In study **III**, 12 separate treatments were applied at two-weekly intervals between May 2 and October 12 in 2005. A total of 144 circular experimental plots were established during the summer and consisted of four replicate plots of each treatment per application date. Three different types of plots were established on each treatment date after cutting the stumps: *C. purpureum* inoculum treatment, a blank inoculum (formulation control) and no inoculum (slash control). The experimental design in **III** was completely randomized. Plots were defined as in **II**, but plot diameter was determined by the area required to include at least 20 birch stumps each with a diameter of more than 10 mm.

The birches were cut with a clearing saw approximately 30 cm above ground level (**II**, **III**) and inoculated within 10 minutes. The freshly cut surface of each stump was entirely covered with approximately 2 ml of inoculum delivered by a plastic squirt bottle.

In **IV**, pruning and inoculations were conducted on seven dates each separated by approximately 1 month between April 14 and October 12 in 2005. On each date, 30 trees were pruned with a pruning saw to yield a total of 210 trees tested. The experimental design was completely randomized. The border of the dead and living branches was marked by cutting off the lowest living branch to leave a long stub. For 10 of the 30 trees pruned, the entire surface of each freshly pruned wound was covered with 1-2 ml of *C. purpureum* inoculum delivered via spray bottle. The fresh wounds of 10 other pruned trees were inoculated with blank inoculum and the remaining 10 trees were only pruned.

3.5.4 Measurements

One stump in each plot was cut at the root collar 3 months after the experiment was established (**II**). To measure the differences in growth rate of the various *C. purpureum* isolates in the field, mycelia was isolated from stumps as described elsewhere (**II**). Stump sprouting and fructification was examined during the first and second growing seasons after treatment; the number of fresh fruiting bodies of *C. purpureum* was calculated on each stump and the number of living sprouts per stump and the height of the tallest living sprout were measured.

The presence of fruiting bodies was examined during the first three autumns following treatment (**III**). Sprouting of stumps (the number of living stumps, the number of living sprouts per stump and the height of the tallest living sprout) was examined in the first and second years following treatment. Weather data during the experimental period (summer 2005) were collected at the Hyytiälä weather station of the Finnish Meteorological Institute, located 19.3 km north of the experimental site.

A total of 129 trees were felled and surveyed 2 years after the experiment was established (**IV**). Felled trees were divided into two different groups depending on their subsequent analysis: 87 trees as so called stem sample trees and 42 trees as branch sample trees. Discs were cut from stem sample trees at 50-cm intervals from ground level up to a maximum of 350 cm. The stage of decay was defined from each disc using the classification explained in **IV**.

The percentage of the discoloured/decayed area of the disc was calculated with a Planimeter Tamaya Digitizing Area-line Meter PLANIX10S “Marble” (Tamaya Technics Inc., Japan). *Chondrostereum purpureum* was isolated from discoloured/decayed areas in the discs and its presence was identified microscopically according to Eriksson and Ryvander (1976). From the branch sample trees, discs were cut at the point of the pruning wounds and as many discs as possible were cut from each stem. The diameter of the pruned branches and spreading of discoloration/decay through the pruning wounds were recorded in the laboratory.

3.5.5 Data analyses

The data from **II**, **III** and **IV** were analysed with SPSS software version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and are outlined in Table 1.

Table 1. Analyses used in various studies.

Analyses	Studies
One-way ANOVA	II
Duncan multiple range test	II
Spearman's correlation coefficient	II
Two-factorial ANOVA	III, IV
Tukey's post hoc test	III, IV

4 RESULTS AND DISCUSSION

4.1 Potential use of *C. purpureum* as a biocontrol agent in Finland

Results showed high potential of use *C. purpureum* as a biocontrol agent in Finland. The genetic variability of *C. purpureum* population is great in Nordic and Baltic countries (I) and the extant diversity likely contains isolates that are suitable for use in biocontrol programs.

The application of *C. purpureum* prevented sprouting of birch efficiently (II, III). Both the frequency of living stumps and the number of living sprouts per stump were significantly reduced following *C. purpureum* treatment (II, III). This finding is in line with Jobidon's (1998) results that showed how *C. purpureum* has a strong potential for stump-sprouting control in *Betula papyrifera*. Although application of the fungus decreased the percentage of stumps with sprouts and the number of living sprouts per stump, it had no effect on the maximum height of new sprouts (II, III). Similarly, Jobidon (1998) and Dumas et al. (1997) found that although application of the fungus decreased the percentage of stumps with sprouts, it did not affect their maximum height in aspen.

Clear differences were found between isolates of *C. purpureum* in preventing sprouting and in forming fruiting bodies on birch (II). One isolate (3.11) clearly produced more fruiting bodies than others; 14 weeks after treatment fruiting bodies were found in almost 80% of stumps treated with this isolate whereas some other isolates produced fruiting bodies in as few as 17.5% of stumps (II). One of the eight isolates tested (HY4) differed clearly in its ability to kill the stumps; two growing seasons after the treatments 92% of the treated stumps were dead whereas 63–85% of stumps treated with other tested isolates had died. In comparison, 44–58% of control stumps died within 2 years of treatment. Stumps treated with isolate HY4 also had the fewest living sprouts per stump; after 2 years the treated stumps had 0.3 sprouts per stump, whereas slash control stumps had 1.5 and formulation control stumps had 2.4 sprouts (II). Results of study II are in accordance with earlier work in that there are differences in virulence and the ability to inhibit stump sprouting among *C. purpureum* isolates (Bennet 1962, Ekramoddoullah et al. 1993, Wall et al. 1996, Pitt et al. 1999, Harper et al. 1999).

To maximize its efficacy as a biocontrol agent, *C. purpureum* must be applied as the right time. The effect of the *C. purpureum* treatment was greatest in May through July and less effective towards the end of the growing season (III). For example, when the treatment was applied in mid-July, only 12.5% of the treated stumps showed resprouting 2 years after treatment compared with 74% of control stumps. When the treatment was applied in late summer, the effect of the treatment improved during the second year (III). Jobidon (1998) found that the effect of the treatment improved similarly during the second year on *Betula papyrifera* and *Prunus pensylvanica*, indicating that control of stump sprouting with *C. purpureum* is a slow-acting process. Wall (1990) stated that the speed of stump invasion by the fungus and reduction of sprouting varied both among and within species. Treatments in late September- October were ineffective since they did not significantly differ from the controls (III).

Results of the study III are in accordance with earlier findings in that the resistance of beech and birch decreases towards midsummer and increases at the end of the growing season (Wall 1991). Also Spiers et al. (1998) showed that the xylem tissue of several hardwood species (*Malus*, *Pyrus*, *Prunus* and *Salix*) exhibits a similar seasonal variation to infection by *C. purpureum*. All hosts showed maximum resistance during midwinter and susceptibility increased in spring/early summer, declining through the late summer and autumn (Spiers et

al. 1998). According to our results, pruning wounds of birches were most susceptible to *C. purpureum* infection in May (IV). This agrees with the results of study III, which showed that early summer is the optimal time for successful infection of birches with *C. purpureum*. With these results in mind, the importance of correct application time is stressed to ensure the maximal effect on sprout prevention.

According to the results presented here, there were no clear connections between the air temperature and the efficacy of *C. purpureum* treatment (III). Neither drought nor light rain around the treatment date seemed to affect the efficacy (III). Earlier studies did not consider the effect of local weather directly although Spiers and Hopcroft (1988a) found that germ tube extension increased dramatically above 17.5 °C but declined rapidly to 0 at 38 °C. Similarly, Wall (1986) found that 37 °C for 7 days was lethal to the mycelium of *C. purpureum*. Fungal formulation increased the tolerance to high temperature and a stumps surface temperature of 43 °C could not kill the fungus as shown by Dumas et al. (1997).

Similar to the findings of De Jong et al. (1996), fruiting bodies of *C. purpureum* were established only a few months after application (II, III). When treatments were applied between May and October in 2005, fructification was most abundant one year later and most fruiting bodies were found on stumps treated in May-July (III). In study II, where treatments were conducted in June 2006, fructification peaked 14 weeks after the inoculation. Additionally, Becker et al. (2005) found that the peak production of *C. purpureum* basidiocarps occurred approximately 22 months after the trial was established, so this aspect of the *C. purpureum* life cycle appears to be quite variable. Rainfall and relative humidity are the key factors for formation of sporophores and basidiospore release (Dye 1974, Spiers 1985, Spiers 1988b), so perhaps differences in relative humidity may explain the variation in emergence of fruiting bodies (II, III). In general, most stumps fructified in 2 consecutive years after the treatment (II, III). This pattern was also observed by Wall (1997).

Sprouting ability of *Betula pendula* and *B. pubescens* varies (Perala and Alm 1990, Johansson 1992, Johansson 2008). In our experimental plots (II, III), at least 75% of stumps were *B. pendula*. However, two birch species were considered together in II and III because it was not possible to reliably separate *B. pendula* from *B. pubescens* stumps. Stumps were identified several months after cutting, after which time the discrimination of these two species was no longer possible, especially in small nonsprouting stumps. The two birch species were also considered together from a practical point of view; they usually grow mixed in the forest and both cause problematic sprouting. Therefore, if *C. purpureum* is to be used as a future biocontrol agent in Finland, it should diminish sprouting in both species.

4.2 The mode of action of *C. purpureum* in preventing stump sprouting of birch

Based on the results of enzyme activity trials, sprouting inhibition may be associated with 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) radical-generating enzymes and Poly-R bleaching (II). Such activities are common in lignin-degrading white-rot basidiomycetes and are mainly due to laccase and/or manganese peroxidases (Hofrichter and Fritsche 1996). Four of eight isolates possessed high ABTS radical cation formation efficiency and Poly-R bleaching on Petri plates and efficiently inhibited sprouting in the field, whereas three could not effectively prohibit sprouting and possessed only very weak oxidative activity towards ABTS and Poly-R dye (II). This may have resulted from microbial oxidative activity, including reactive oxygen species production that elicited a hypersensitive response in plants and initiating a cascade of reactions that diminished sprout production.

However, only 8 isolates were field-tested in study **II** so additional experiments are needed to test the hypothesis that sprout inhibition is directly related to the high oxidative activities of *C. purpureum*. The virulence of *C. purpureum* may also be dependent on other factors that were not considered here, e.g., silver-leaf disease in fruit trees is caused by phytotoxins released by *C. purpureum* (Bishop 1979).

High *in vitro* cellulase, lipase or protease activity did not correlate with efficiency of sprout control in the field. Prior to completing study **II**, we expected pectin-degrading activity to be a crucial factor in the inhibition of sprouting since endopolygalactouronase activity is a factor of silver-leaf disease symptoms in apple seedlings (Miyairi et al. 1985). Therefore, it was somewhat surprising that none of the 21 *C. purpureum* isolates showed high pectin-degrading activity on nutrient agar plates (**II**). However, the lack of pectinase activity did not affect the ability of *C. purpureum* to infect fresh birch wood or kill the stump. While it must be remembered that the qualitative enzyme assay in study **II** can be rather subjective, the same individual conducted all visual assessments and, in most cases, alternative results were distinct. This approach enabled us to screen large numbers of isolates and several enzyme systems (**II**).

There were differences among isolates of *C. purpureum* with respect to the growth of mycelial biomass on wood chips in the laboratory (**II**). However, variation in media quality among replicates did not allow clear conclusions to be made on the growth capability of different isolates of *C. purpureum*. Further studies are required to evaluate the correlation between fungal growth and efficiency of sprout control.

4.3 Environmental risks of using *C. purpureum* for biocontrol purposes in Finland

Chondrostereum purpureum showed high genetic diversity among (1.2%) but almost no differentiation within (98.8%) populations in its Nordic and Baltic range (**I**). This structure implies that biocontrol programs using local strains can do so without fear of introducing new genotypes to treatment areas. Similarly, Ramsfield et al. (1999) studied mtDNA variation among population samples from New Zealand, Europe and North America and found that 96.5% of the variability occurred within subpopulations, while 3.5% occurred among subpopulations.

When the risk of incidental infection of nontarget trees was evaluated, results showed that pruning wounds of birches were most susceptible to infection by *C. purpureum* in early spring (**IV**). Decay was most often found and the proportion of the discolored or decayed area in the cross-section of the stem was highest when trees were pruned in May. Spreading of the discoloration/decay from pruning wounds to living tissue was also highest when the trees were inoculated in May. The results are in accordance with those of Brooks and Moore (1926), who obtained infection of *Prunus* spp. twigs more easily in late winter and early spring than in summer. In contrast, the resistance of yellow birch to invasion by the fungus is greatest in spring and decreases towards midsummer, after which it appears to increase again (Wall 1991).

When evaluating risks to nontarget trees, it must be borne in mind that the artificial inoculation method used here (**IV**) is very different from the natural situation: natural infection of *C. purpureum* occurs via airborne basidiospores but in study **IV** the mycelium was placed directly on freshly-pruned wounds. A greater depth of penetration was obtained using mycelium as the inoculum rather than basidiospores (Spiers and Hopcroft 1988a).

This suggests that the natural spread of *C. purpureum* may not cause as severe a level of discoloration/decay in pruned birches as was shown in the present study (IV).

Incidental infection attributable to the use of *C. purpureum* as a biocontrol agent is dependent on many factors. Firstly, pruning wounds of nontarget trees must be freshly made; Brooks and Moore (1926) showed that it was difficult to infect month-old wounds and impossible to infect 3-month-old wounds. Also, incidental infection of nontarget trees can only take place via spores, when *C. purpureum* is fructificating and sporulating on treated stumps. Sporophores are established only a few months after treatment and *C. purpureum* usually fructificates continuously on the same host for up to 2 years (Wall 1997, II, III). Finally, nontarget infection risk decreases with distance from the treatment site, i.e., high at 500 m from the control area but negligible 5000 m distant (De Jong et al. 1990b).

5 CONCLUSIONS AND FUTURE PROSPECTS

Results of this study indicate that the application of *C. purpureum* after manual cutting reduces the sprouting of birch in myrtillus type forests of central Finland. The treatment was shown to be effective in mixed silver and downy birch stands on stumps of 3-4 cm diameter. Before these implications are generalized, additional research is necessary to determine the efficacy of *C. purpureum* treatment on other tree species and on stumps of different diameters than those considered here. Furthermore, the site used in this study is more similar to a forested situation rather than roadsides or under power lines (where conditions can be quite different) so this research should be repeated in diversity habitats and different parts of Finland.

Different isolates of *C. purpureum* exhibited clear differences in the ability to prevent sprouting. As such, one of the directions of future research will be to identify an optimal isolate(s) for use in biocontrol programs. Oxidative enzyme activity appears to play a role in sprout inhibition, although rather few isolates tested (n=8) limit the extent to which this result can be considered robust. The pathogenic mechanism of *C. purpureum* is likely dependent on many factors, e.g., phytotoxins, and has not yet been fully elucidated. Unfortunately, there is no rapid *in vitro* protocol to screen high numbers of isolates for use in biocontrol programs. Development of such a protocol is a research priority, but in order to be successful it must integrate pathology, virulence, lab vs. field performance as well as genetic and environmental factors. As such, this research directive is an ambitious one indeed.

Timing of application clearly affects the efficacy of *C. purpureum* treatment. Treatment effect was greatest when applied in early and mid summer and less effective towards the end of the growing season. However, when the treatment was performed in late summer the effect improved during the second year. In late autumn (late September-October) the treatment was not effective at all. Although the percentage of living stumps and the number of living sprouts per stump decreased after treatment, it had no effect on the maximum height of sprouts. In conclusion, the most efficient time for *C. purpureum* treatment is early and midsummer. However, since pruning wounds of birches are most susceptible to infection of *C. purpureum* at that time, pruning of nontarget trees should be avoided in close proximity to the treatment area.

Selective culture may improve the performance of this fungus in controlling sprouting and would be a useful way to obtain more efficient isolates of *C. purpureum* for use as biocontrol agents. Selective breeding has been widely used to improve the properties of domesticated animals and plants and the approach should also be considered for commercially applied fungi. We found several isolates that were clearly more effective at preventing sprouting than others. If these isolates could be cross-bred, their progeny could be even more efficient at preventing sprouting than the isolates used in here.

The pathogenicity of *C. purpureum* towards different broad-leaved tree species is variable (e.g., Pitt et al. 1999). It would also be useful to test the efficacy of Northern European isolates on broad-leaved trees other than birch. To fully develop *C. purpureum* treatment as an operational tool in vegetation management, isolates that would efficiently prevent sprouting of all problematic tree species should be found. Thereafter, *C. purpureum* treatment could play an important future role in integrated forest management in Finland, especially in areas where only a few wounded non-target broad-leaved trees are present and conifers are the main crop species.

Chondrostereum purpureum is a pioneer colonizer of woody substrates. It has a low competitive ability and is quickly replaced by secondary fungi (Rayner and Boddy 1986).

Unfortunately, we did not examine naturally-occurring decomposer fungi or other fungi/bacteria from the inoculated stumps. Therefore, we cannot consider the extent to which other decay fungi or microbes are involved in stump decay or the inhibition of resprouting. However, it would be worthwhile to follow the succession of fungi on inoculated stumps and determine if and how they affect resprouting.

The use of biological control agents always requires permission. Without a registered formulation of *C. purpureum*, it cannot be used as an operational tool for controlling sprouting in Finland. The registration process for biocontrol agents is expensive, time-consuming and laborious, since it requires many evaluations and extensive risk assessments. This study showed that the potential environmental risks posed by use of *C. purpureum* treatment appear to be quite small and encourage continued research in this respect.

The genetic data showed high diversity among the Nordic and Baltic populations of *C. purpureum* but almost a complete lack of local differentiation. This implies that any local strain from the area can be used as a biocontrol agent without fear of introducing new genotypes to the treatment areas. Pruned silver birches are most susceptible to infection by *C. purpureum* in early spring. Therefore, there may be an increased threat to pruned silver birches in early spring if they are located close to an area where *C. purpureum* has been recently used for biocontrol purposes. However, the artificial inoculation used in this study is very different from the natural mode of infection in which *C. purpureum* is distributed by spores.

Use of *C. purpureum* as a biocontrol agent on a large scale will require application technology to be developed and placed in series following mechanical cutting machinery. In forestry, one possibility would be to combine *C. purpureum* application and final cutting. Then, it would be possible to apply *C. purpureum* with the same technique used to control *Heterobasidion* root rot, the most important disease of conifers in northern temperate forests (Pratt et al. 1998).

Although *C. purpureum* application clearly reduces the percent of living stumps and the number of sprouts per stump, it had no effect on the maximum height of sprouts. This may be a problem for *C. purpureum* as a biocontrol agent in practice. When evaluating the need for sprout control, both number and height of sprouts are the critical factors. It must be considered whether the total reduction in sprouting and re-growth due to *C. purpureum* treatment is sufficient to be of economic benefit. Economic factors such as manufacturing and distribution costs, equipment purchases, and costs associated with storage and application will also determine whether *C. purpureum* is adopted as a management tool in future.

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